Triptolide inhibits lipopolysaccharide-induced proliferation, induces apoptosis and causes cell cycle arrest of human mesangial cells in vitro

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Abstract: Triptolide, an important active ingredient of the traditional Chinese medicine *Tripterygium wilfordii* Hook F, has been extensively researched. In the present study, we investigated the effects of triptolide on lipopolysaccharide (LPS)-induced proliferation of human mesangial cells in vitro and the underlying mechanisms. Human mesangial cell proliferation was detected by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays. Morphological changes of human mesangial cells were observed under an inverted microscope, and cell ultrastructural alterations were assessed by transmission electron microscopy. Apoptosis was evaluated by Annexin V-FITC/PI staining. Flow cytometry was used to detect cell cycle changes in treated cells. Expression of α-SMA in human mesangial cells was detected by immunofluorescence and reverse transcription-polymerase chain reaction (RT-PCR). In addition, RT-PCR and Western blot analyses were conducted to determine transforming growth factor (TGF)-β1 levels in human mesangial cells. Smad2, p-Smad2, Smad3, and p-Smad3 protein levels were detected using Western blotting. MTT assays showed that treatment with LPS significantly promoted human mesangial cell proliferation. Triptolide significantly inhibited LPS-induced proliferation, promoted cell apoptosis, and caused cell cycle arrest in *G₀/G₁* phase in human mesangial cells. The mRNA levels of α-SMA and TGF-β1 were significantly reduced following triptolide treatment. Furthermore, the levels of TGF-β1, p-Smad2 and p-Smad3 proteins were significantly decreased in triptolide groups in a dose-dependent manner. In conclusion, triptolide inhibits proliferation, induces apoptosis and causes cell cycle arrest in human mesangial cells, possibly by blocking the TGF-β1/Smad signaling pathway and down-regulating α-SMA expression.

Keywords: Triptolide, human mesangial cells

Introduction

Triptolide, an active ingredient of the traditional Chinese medicine *Tripterygium wilfordii* Hook F (TwHF), has positive therapeutic effects, including immunosuppressive, anti-inflammatory, and anti-cancer properties and protects against acute cerebral ischemia/reperfusion (I/R) injury [1-3].

Triptolide has been shown to be useful in preventing kidney transplant rejection, and it was found to exhibit a prominent anti-albuminuric effect on diabetic nephropathy substantially attenuating albuminuria and podocyte injury [4-7]. Although the inhibitory role of triptolide in pulmonary fibrosis and its antifibrotic effect have been well studied, its function in the inhibition of mesangial cell proliferation and prevention of renal fibrosis requires further research [8, 9]. Mesangial cells, vital intrinsic glomerular cells, are involved in maintenance of glomerular ultrafiltration. Research has shown that glomerulonephritis is the primary cause of end stage renal disease (ESRD), and mesangial proliferative glomerulonephritis is the most common type of glomerulonephritis [10, 11]. Superfluous proliferation of mesangial cells and extracellular matrix (ECM) accumulation is the cause of glomerular sclerosis, and a previous study examining human mesangial cell proliferation-related factors found a significant association with alleviation of glomerular injury [12]. Apoptosis plays an important role in eliminating excessive proliferation of human mesangial cells in the kidney disease re-
pair process [13, 14]. Lipopolysaccharide (LPS), a major endotoxin located in the cell wall of gram-negative bacteria, is a key factor triggering many detrimental physiological processes.

Mesangial cells are associated with various pathological stimuli of glomerular inflammation, including cell proliferation, fibrosis, and leukocyte infiltration, which are primarily initiated by adhesion molecules [15, 16]. In bacterially induced glomerulonephritis, LPS induces VCAM-1 in the murine glomerular mesangium [17].

The present study evaluated the effects of triptolide on LPS-induced human mesangial cell proliferation and elucidated the underlying mechanisms. The findings offer a theoretical foundation for the extensive application of triptolide in clinical practice.

Materials and methods

Cell culture

Human mesangial cells lines were purchased from the American Type Culture Collection (ATCC, USA). The cells were cultured in RPMI-1640 medium in tissue culture flasks. The medium was supplemented with 10% fetal bovine serum (FBS), and human mesangial cells were cultured at 37°C under a humidified atmosphere of 5% CO₂ and 95% air.

Reagents and chemicals

Triptolide and LPS were purchased from Sigma (St. Louis, MO, USA). MTT was purchased from Beyotime (Shanghai, China). Triptolide was dissolved in dimethyl sulfoxide (DMSO), with the DMSO concentration not exceeding 0.01%, and was added directly to the culture medium of human mesangial cells were cultured at 37°C under a humidified atmosphere of 5% CO₂ and 95% air.

MTT assay

Assessment of the effect of triptolide on human mesangial cells by the MTT colorimetric method. Fully differentiated human mesangial cells were digested using 0.25% trypsin, and the human mesangial cells were inoculated in 96-well plates at an inoculation density of 1 × 10⁵ cells/well and an inoculation volume of 80 µL/well. The volume of solution in the wells was adjusted to 100 µL/well with culture medium. The inoculated cells were cultivated in an incubator at 37°C containing 5% CO₂ until they reached confluency. Triptolide solutions at concentrations of 0, 5, 10, 20, 40 and 60 ng/ml were added to different wells (n = 6 replicate wells/concentration). The plates were placed in the incubator for 24 h, and then 10 µL MTT solution was added to each well for another 4 h, after which the cultivation was stopped. The supernatant in each well was pipetted away, 150 µL DMSO was added to each well, and the plates were shaken for 10 min to fully dissolve. The solution was subjected to triplicate optical density (OD) measurements at a wavelength of 490 nm on an ELISA reader (Bio-Rad, Hercules, CA), with a triptolide solution without cells serving as a blank reference and a mixture of culture medium and DMSO serving as a baseline reference. The human mesangial cells survival rate was defined as (drug group OD - baseline OD)/ (blank reference OD - baseline OD) × 100%.

Based on the results of the MTT experiments described above and the references, we selected 10, 20 and 40 ng/ml dose of triptolide as the concentration of subsequent experiment.

Then, the samples were randomly divided into the following five groups: (1) Normal control group (Control group, containing 10% FBS), (2) LPS group (10 µg/mL LPS group), (3) Triptolide low dose group (10 ng/ml TP group, 10 µg/mL LPS and 10 ng/ml TP), (4) Triptolide medium dose group (20 ng/ml TP group, 10 µg/mL LPS and 20 ng/ml TP), (5) Triptolide high dose group (40 ng/ml TP group, 10 µg/mL LPS and 40 ng/ml TP).

Analysis of morphological changes

Human mesangial cells in the logarithmic phase were plated in a 6-well culture plates at a density of 1 × 10⁵ cells/ml in culture medium.
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Table 1. The sequence and length of the primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Length (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>F GACTACTAGCGCAAGGAGGTAC</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>R GAGCAACACGCGGTCCAGTA</td>
<td>20</td>
</tr>
<tr>
<td>α-SMA</td>
<td>F TCTTCCAGGCTTCTTAC</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>R TTGGCATACAGGTCCTTC</td>
<td>20</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F GCACCGTCAAGGCGGAGA</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>R TGGTGAAGACGCCAGTGA</td>
<td>19</td>
</tr>
</tbody>
</table>

The cells were cultured overnight and then treated with LPS and different doses of triptolide for 24 h. Then, we observed the morphological changes in the human mesangial cell by an inverted microscope.

Transmission electron microscopy

Cells were treated as described above for 24 h or established as controls. At the end of the treatment, the cells were washed twice with phosphate buffered solution (PBS, 137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L NaHPO₄, 1.4 mmol/L KH₂PO₄, pH 7.4), fixed in 2.5% (v/v) glutaraldehyde followed by 1% (v/v) perosmic acid, and dehydrated in an ethanol series. Ultrathin sections were placed on 400-mesh grids and double-stained with uranyl acetate and lead citrate. The ultrastructures were observed using a transmission electron microscope (HITACHI-H7650, Tokyo, Japan).

Cell apoptosis assay

Cell apoptosis was detected by flow cytometry using the Annexin V: FITC Apoptosis Detection Kit II (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Briefly, cells were washed twice with cold PBS and resuspended in binding buffer. To each well of a 96-well plate, and 2 μL of Annexin V-FITC was added to 100 μL of cell suspension (1 x 10⁵ cells) for 15-min incubation on ice in the dark. Before detection, 400 μL of PBS and 1 μL of propidium iodide (PI) were added. Flow cytometry (BD Biosciences) was used to detect the FITC-positive and PI-negative cells (early apoptotic), which were regarded as apoptotic cells in the analysis.

Cell cycle analysis

The effects of triptolide treatment on cell cycle distribution in human mesangial cells were analyzed using PI staining (SigmaAldrich, St. Louis, MO, USA) and flow cytometry (Cytomics FC 500; Beckman Coulter, Inc., Brea, CA, USA). A total of 1 x 10⁶ human mesangial cells were seeded in each well. The cell treatments and groups were the same as those previously described for the MTT assay, and 70% alcohol was used as a fixative. Cells were collected, and we added 5 μL Annexin V/5 μL FITC and 10 μL PI for 30 min in the dark. Cells were detected within 1 h by flow cytometry, and cell cycle analysis was performed using the FlowJo software to calculate the apoptosis cell percentage.

Immunofluorescence microscopy

Human mesangial cells mounted on glass slides were fixed with 4% paraformaldehyde for 20 min and permeabilized with PBS containing 0.1% Triton X-100 and 0.1% glycine for 2 min on ice. TUNEL staining was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Germany). Cells were costained with 4',6-diamidino-2-phenylin-dole (DAPI) to visualize the nuclei. The images were taken using a FSX100 all-in-one microscope (Olympus Corporation, Japan).

Real-time PCR

Total RNA was isolated from the human mesangial cells using TRIzol reagent (TransGen Biotech, Beijing). The conversion of 1 μg of to-
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In this study, RNA was converted into cDNA using a commercial kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. Primers for human TGF-β1, α-SMA, and GAPDH were designed by TaKaRa Biotechnology (Table 1). Real-time PCR was performed in a 20 µL reaction mixture containing 10 µL 2 × SYBR Green master mix, 7 µL ddH2O, 2 µL primers (10 µM), and 1 µL template DNA. Cycle conditions were obtained from the manufacturer’s instructions. A fluorescence quantitative PCR instrument (Bio-Rad, CFX-96, CA, USA) and a SYBR® Premix Ex Taq™ kit (TaKaRa) were used to detect target gene expressions, and GAPDH was used as an internal reference. The 2^−ΔΔCT method was employed to determine the relative expression of target genes normalized to that of GAPDH, and experiments were conducted in triplicate.

**Western blot**

After the experiment, human mesangial cells were lysed with cell lysis buffer on ice for 30 min. After centrifugation at 20,000 × g for 20 min at 4°C, the nuclear TGF-β1, Smad2, P-Smad2, Smad3, and P-Smad3 proteins were extracted using the Pierce NE-PER kit (Pierce, Rockford, IL, USA). A BCA protein assay kit was used to measure the protein concentration. Samples were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane in a semi-dry system (Bio-Rad). Then, the membrane was blocked with 5% fat-free milk in TBST buffer (20 mmol/L Tris-HCl, 137 mmol/L NaCl and 0.1% Tween 20) and incubated with primary antibodies against TGF-β1, Smad2, p-Smad2, Smad3, and p-Smad3 (1:1000) in TBST buffer overnight at 4°C. After 3 washes in PBST, the membrane was incubated in horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Signals were developed using the EasyBlot ECL Kit (Sangon Biotech, Shanghai, China), and the grey-scale value of the tested proteins was analyzed using the Gel-Pro Analyzer v4.0 (Media Cybernetics, L.P.) and compared to that of β-actin.

**Statistical analysis**

All experiments were conducted with 3 replications, and the data are expressed as the mean ± standard deviation of the samples. Differences between groups were assessed by Student’s t-test and one- or two-way ANOVA with Duncan multiple comparisons using SPSS 18.0 (SPSS, Chicago, IL, USA). A value of P<0.05 was considered statistically significant.

**Results**

**Effect of triptolide on human mesangial cells viability**

Human mesangial cells treated with triptolide at different concentrations for 24 h showed decreased viability with increasing triptolide concentrations. In particular, the human mesangial cells viability decreased to 71.34 ± 3.12% after a 24 h treatment with 60 µg/ml triptolide, whereas 24 h treatments with 5-40 ng/ml triptolide did not significantly affect human mesangial cells viability (Figure 1).

**Inhibition of LPS-induced cell proliferation by triptolide treatment**

We observed that exposure of human mesangial cells (HMCs) to LPS significantly enhanced cell proliferation (P<0.05). However, triptolide inhibited cell proliferation in a dosedependent manner. Cells were exposed to different doses of triptolide to investigate the effect, and we observed that 10, 20, and 40...
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Figure 3. The morphological changes of human mesangial cells were observed under an inverted microscope. Magnification ×200. Comparison of the cell morphology of human mesangial cells among the five groups. A: Control; B: LPS (10 μg/mL); C: TP (10 ng/mL); D: TP (20 ng/mL); E: TP (40 ng/mL).

Figure 4. Comparison of human mesangial cells among the five groups by transmission electron microscopy. A: Control. B group: LPS (10 μg/mL). The treatment groups [C: TP (10 ng/mL); D: TP (20 ng/mL); E: TP (40 ng/mL)] displayed prominent myofilament disarray and rupture, cytoplasmic vacuolization, significant mitochondrial swelling and apoptotic bodies formed. The upper scale bar = 5 μM, magnification ×4000; the middle scale bar = 2 μM, magnification ×10000; and the lower scale bar = 1 μM, magnification ×30000.

ng/ml triptolide for 24 h led to 17.23 ± 3.45%, 28.05 ± 3.63%, and 39.87 ± 2.15% reductions, respectively, in cell viability (P<0.05) (Figure 2A and 2B).
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Figure 5. Induction of apoptosis of human mesangial cells by triptolide and analysis of cell viability (A, B). *P<0.05 vs control, **P<0.05 vs LPS.

Morphological changes of HMCs

The results showed that compared with the blank control group, cells in each treatment group appeared to have a different degree of morphological change, as based on inverted phase-contrast microscopy. In the triptolide treatment groups, some cells changed from an irregular elliptical shape to a circular morphology, and the morphology changes became more notable with increasing drug concentration (Figure 3).

Transmission electron microscopy

Transmission electron microscopy was then used to investigate the ultrastructure of apoptotic cells. The cells in the control group were round with tiny villous projections of the cell membrane. Many plasmosomes were distributed in the nucleus, and the structure of the mitochondria was clear; the rough endoplasmic reticulum was streaky, and lipid droplets were found in the cytoplasm. In the triptolide treatment groups, HMC growth was clearly in-
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Figure 6. Cell cycle analysis of each group by flow cytometry (A, B). *P<0.05 or **P<0.01 vs control, *P<0.05 or ***P<0.01 vs LPS.

Induction of HMC apoptosis by triptolide

The results revealed that exposure of HMCs to triptolide significantly enhanced apoptosis (*P<0.05). Treatment with FBS, LPS and 10, 20,
and 40 ng/mL triptolide for 24 h resulted in (9.23 ± 1.86)%, (3.34 ± 1.36)%, (11.49 ± 2.40)%, (18.06 ± 2.20)%, and (26.02 ± 2.37)% apoptosis, respectively, in each group of cells (Figure 5A and 5B).

**Induction of the cell cycle in HMCs by triptolide**

Flow cytometry showed that LPS could promote HMC from G₀/G₁ phase to S phase, however, after triptolide treatment, the proportion of cells in S phase decreased gradually, the proportion of cells in G₀/G₁ phase gradually increased, mitotic cells were arrested in G₀ phase (p<0.05 or p<0.01) (Figure 6A and 6B).

**Immunofluorescence in HMCs**

To further elucidate how triptolide prevents renal interstitial fibrosis, we investigated its ability to regulate HMC function. Double immunofluorescence staining was performed using antibodies directed against α-SMA, and the results indicated that triptolide down-regulated α-SMA in these cells. Additionally, α-SMA was significantly higher in the LPS group compared with the normal group. However, after triptolide treatment for 24 h, the levels of α-SMA expression decreased significantly at all doses (Figure 7A, 7B).

**Inhibition of TGF-β1, Smad2, p-Smad2, Smad3 and p-Smad3 expression by triptolide**

The effect of triptolide on TGF-β1, Smad2, phospho (p)-Smad2, Smad3 and p-Smad3 expression in LPS-treated HMCs was also investigated. Elevation of TGF-β1 expression due to LPS in HMCs was significantly reduced by triptolide, and similar changes were found for the
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**Figure 8.** Quantification of the mRNA expression of TGF-β1 (A). Western blot analysis results showing the TGF-β1, Smad2, p-Smad2, Smad3 and p-Smad3 protein levels in each group (B) and Western blot results showing protein levels of TGF-β1 (C), p-Smad2/Smad2 (D), p-Smad3/Smad3 (E). *P*<0.05 or **P*<0.01 vs control, *P*<0.05 or **P*<0.01 vs LPS.

levels of p-Smad2 and p-Smad3 (*P*<0.05 or *P*<0.01). In contrast, triptolide downregulated TGF-β1, p-Smad2 and p-Smad3 protein expression in a dose-dependent manner (Figure 8A-E).

**Discussion**

Triptolide is an active component of TwHF. In addition to its classic immunosuppressive properties [18], triptolide causes cell cycle arrest, inhibits cell proliferation, induces apoptosis, reduces proteinuria, and accelerates glomerular injury repair during the treatment of rats with nephritis [19]. All these effects are gaining increasing attention. Under normal physiological conditions, glomerular mesangial cell proliferation and apoptosis are in dynamic equilibrium. However, following exposure to exogenous inflammatory mediators, cytokines can cause mesangial cells to proliferate and the extracellular matrix (ECM) to accumulate, leading to glomerular sclerosis, renal fibrosis and other kidney diseases [20, 21].

Cell proliferation is achieved through the cell cycle. As the cell cycle progresses, cells in G1-S phase enter G2 phase, and mitosis occurs in M phase. Our study showed that LPS significantly promoted the cell cycle compared with the control groups and that treatment with different concentrations of triptolide caused cell cycle arrest at G1 phase [22]. This phenomenon indicates that LPS can induce human mesangial cell proliferation by promoting progression of the HMC cycle and that triptolide can block the cell cycle at G1 phase, inhibiting proliferation. Apoptosis is characterized by specific biochemical and morphological changes, and many methods have been developed to assess apoptosis, such as flow cytometric analysis, morphological studies, and biochemical assays. In the present study, typical morphological changes were observed following exposure to different concentrations of triptolide. We found that triptolide inhibited proliferation and induced apoptosis in HMCs. Efficient inhibition of mesangial cell proliferation and induction of apoptosis would decrease the progression of glomerular sclerosis and chronic glomerulonephritis.

Studies have demonstrated that TGF-β1 plays an important role in mesangial cell injury and glomerular sclerosis [23, 24]. Many growth cytokines and signaling pathways are involved in the process of mesangial fibrosis. TGF-β1 is the most versatile, with roles in cell growth, proliferation, apoptosis, and ECM production [25, 26]. The underlying molecular mechanisms of the beneficial effects of triptolide include suppression of the TGF-β1/Smad signaling pathway [27] and inhibition of protein α-SMA expression [28]. Numerous studies have con-
firmed that TGF-β1 has an important role in renal fibrosis, predominantly through a Smad signal transduction pathway-dependent mechanism, but the relationship between the TGF-β1/Smad signaling pathway and mesangial cell apoptosis has not been fully elucidated [29, 30]. Our findings show that in vitro levels of TGF-β1, α-SMA, Smad2, and Smad3, and phosphorylated Smad2 and Smad3 were significantly higher in the LPS group than in the control group, suggesting that LPS can stimulate HMC proliferation. After treatment with triptolide, substantial reductions in α-SMA, TGF-β1, Smad2, and Smad3, and phosphorylated Smad2 and Smad3 were observed. Therefore, the effects of triptolide against renal fibrosis in future clinical studies will be examined to determine whether this compound can be applied as a new method for the treatment of renal fibrosis [31].

The results of our study revealed that LPS promotes HMC proliferation and induces α-SMA, TGF-β1, p-Smad2 and p-Smad3 expression. Furthermore, triptolide treatment was shown to inhibit HMC proliferation, induce apoptosis, and cause cell cycle arrest as well as to reduce α-SMA, p-Smad2, p-Smad3 and TGF-β1 mRNA and protein levels. Future studies are required to investigate the mechanisms underlying the effects of triptolide on other signaling pathways.

Triptolide inhibits proliferation, induces apoptosis and causes cell cycle arrest in HMCs, possibly by down-regulating α-SMA and the TGF-β1/Smad signaling pathway.

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Disclosure of conflict of interest

None.

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