Matrine suppresses lipopolysaccharide-induced fibrosis in human peritoneal mesothelial cells by inhibiting the epithelial-mesenchymal transition

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Abstract
Background: Peritoneal fibrosis is the primary reason that patients with end-stage renal disease (ESRD) have to cease peritoneal dialysis. Peritonitis caused by Gram-negative bacteria such as Escherichia coli (E. coli) were on the rise. We had previously shown that matrine inhibited the formation of biofilm by E. coli. However, the role of matrine on the epithelial-mesenchymal transition (EMT) in peritoneal mesothelial cells under chronic inflammatory conditions is still unknown.

Methods: We cultured human peritoneal mesothelial cells (HPMCs) with lipopolysaccharide (LPS) to induce an environment that mimicked peritonitis and investigated whether matrine could inhibit LPS-induced EMT in these cells. In addition, we investigated the change in expression levels of the miR-29b and miR-129-5p.

Results: We found that 10 µg/ml of LPS induced EMT in HPMCs. Matrine inhibited LPS-induced EMT in HPMCs in a dose-dependent manner. We observed that treatment with matrine increased the expression of E-cadherin (F=50.993, P<0.01), and decreased the expression of alpha-smooth muscle actin (F=32.913, P<0.01). Furthermore, we found that LPS reduced the expression levels of miR-29b and miR-129-5P in HPMCs, while matrine promoted the expression levels of miR-29b and miR-129-5P.

Conclusions: Matrine could inhibit LPS-induced EMT in HPMCs and reverse LPS inhibited expressions of miR-29 b and miR-129-5P in HPMCs, ultimately reduce peritoneal fibrosis. These findings provide a potential theoretical basis for using matrine in the prevention and treatment of peritoneal fibrosis.

Keywords: Matrine; Human peritoneal mesothelium cells; Lipopolysaccharide; miR-129-5p; miR-29b

Introduction
Chronic kidney disease is an important global public health problem. Peritoneal dialysis (PD) is a primary method of renal replacement therapy (RRT) in patients with end-stage renal disease (ESRD).[1-3] One of the main complications of PD is peritonitis. The number of Gram-negative bacterial infections stay in a high position. E. coli is the leading cause of Gram-negative peritonitis.[6-7] When acute peritonitis occurs, interleukin-6 (IL-6) induces chemokines such as CXCL8 and CCL2. These recruit monocytes to the peritoneum and directly impact the peritoneal mesothelial cells.[8,9] Long-term chronic peritoneal inflammation induces angiogenesis, changes in the phenotype of peritoneal mesothelial cells, and the deposition of extracellular matrix, eventually leading to peritoneal fibrosis.[10,11]

Peritoneal mesothelial cells are the first line of defense and the physiological barrier in contact with the peritoneal dialysis fluid. LPS is a major component of the outer wall of E. coli and induces epithelial-mesenchymal transition (EMT) in peritoneal mesothelial cells.[12-18] During EMT, the epithelial cells lose adhesion and close connections with adjacent cells and eventually convert to mesenchymal cells. EMT in peritoneal mesothelial cells initiates peritoneal fibrosis and is reversible.[13] EMT is characterized by decreased expression of epithelial cell markers, such as E-cadherin, and increased expression of mesenchymal cell markers, such as alpha-smooth muscle actin (α-SMA).

Yi-Zheng Li and Xi Peng contributed equally to this study.

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Chinese Medical Journal 2019;132(6)

Received: 14-01-2019 Edited by: Li-Min Chen
MicroRNAs are a class of small, non-coding single-stranded RNA molecules composed of about 18 to 25 nucleotides that regulate cellular differentiation, proliferation and apoptosis. MiR-29b inhibits fibrosis by directly targeting the 3’ untranslated regions (UTRs) of several extracellular matrix genes including collagen (COL1A1, COL1A2, and COL3A1), elastin (ELN), fibrillin 1 (FBN1), and the transforming growth factor-β1 (TGF-β1) coding sequence region in exon 3. MiR-29b also reduces the expression of vascular endothelial growth factor (VEGF), inhibits angiogenesis and protects the function of the peritoneum by inhibiting the TGF-β/Smad pathway. Matrine inhibits the metastatic spread of glioma by inhibiting the p38-MAPK and AKT pathways to regulate the EMT process. Matrine inhibits the metastatic spread of glioma by inhibiting the p38-MAPK and AKT pathways to regulate the EMT process. Previous work from our group found that matrine inhibited the formation of E. coli biofilm. We used LPS to simulate an inflammatory peritoneal environment to induce EMT in human peritoneal mesothelial cells (HPMCs). We further explored the effects of matrine on LPS-induced EMT in these cells.

Methods

Reagents

Normal HPMC were purchased from Ji Hi Technology of Guangzhou, China. The cell proliferation and cell toxicity detection kit were purchased from Beibo (Shanghai, China). Rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody and rabbit anti-E-cadherin monoclonal antibody were purchased from CST, and rabbit anti-alpha-SMA polyclonal antibody was purchased from eagle company (Wuhan, China).

Subculture of HPMCs

The HPMCs were cultured for 24 h at 37°C in an environment that was maintained at 95% O₂ and 5% CO₂. When the cells were 80% confluent, the culture medium was replaced. The cells were washed three times with 1× phosphate buffered saline (PBS) followed by digestion with trypsin EDTA (25%). After most of the cells had shrunk and the cell gaps had widened, 2 mL of culture was added to terminate digestion. The cells were centrifuged at 1000 r/min for 5 min and the supernatant was discarded. Then, 2 mL of complete medium was added and the cells were suspended and passaged in a 1:2 to 1:3 ratio. The culture medium was changed to remove dead cells after 24 h.

Counting cells

The cell suspension was added to the edge of the coverslip, and the cells were counted with a counter by observing through an inverted microscope. If the proportion of the cell mass was greater than 10%, the suspension was re-made.

Cell treatments

After the cells were counted, 5 × 10⁴ cells/mL were seeded into cell culture flasks or six-well plates. When the cells were about 70% to 80% confluent, 10 μg/mL of LPS, with or without addition of different concentrations of matrine (0.2, 0.4, and 0.6 μg/mL) were used to stimulate the HPMC for 24 and 72 h.

MTT experiment

HPMCs in logarithmic growth phase were adjusted to 5 × 10⁴ cells/well and were incubated in DMEM containing 10% fetal bovine serum (FBS) in 96-well plates. To the adherent cells, 0, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 μg/mL of LPS were added, in addition to 0, 0.2, 0.5, 1.0, 1.5 and 2.0 μg/mL of matrine. After a 24-h treatment, 5 mg/mL MTT solution was added to each well, and incubated in the dark for 4 h. After solubilization, the optical density (A) was read at 490 nm to estimate the number of viable cells.

Cell scratch repair test

HPMCs at a density of 2 × 10⁵ cells per well were seeded overnight. At 90% confluency, culture medium without fetal calf serum was added to synchronize the cells. After the cells grew to cover the bottom of the plate, the cells were scratched with a sterile pipet tip, and washed three times with 1× PBS. This was followed by adding medium without FBS or LPS, or LPS, and matrine (0.4 μg/mL). The width of the scratch was recorded using an inverted microscope at 0, 8, 16, and 24 h.

Fluorescent quantitative RT-PCR

Total RNA was extracted from HPMC cells with Trizol. In accordance with the instructions of the TAKARA RNA reverse transcription kit. The concentration of the sample was about 500 ng/mL and the purity of A 260/280 nm was between 1.8 and 2.0. First-strand cDNA was synthesized by using oligo-dT primers and reverse transcriptase. The sequence of the primers is as follows: GAPDH, forward 5'-CGGAGTCAACGGATTTGGTCGTAT-3', reverse 5'-AG-CCTTCTCCATGGTGGTGAAGAC-3'; E-cadherin: forward 5'-TTGCTCACATTTCCCAACTCCTC-3', reverse 5'-CACCTTCAGCACCCCATTTGTTTCTC-3'; α-SMA: forward 5'-AGGAAGACAGCACAGCTC-3', reverse 5'-TTACAGAGCCAGCCATT-3'.

We prepared the PCR reaction mix according to the instructions of the 7500 ABI fluorescence quantitative PCR instrument, the reaction condition was performed for 40 cycles. GAPDH was used as an internal control.

Western blotting

The cells were washed three times with cold 1× PBS. This was followed by placing 1× PBS. This was followed by placing 120mL of cell lysate on ice for 15 min, and centrifuging at 12,000 r/min for 15 min at 4°C. The membranes were incubated with the following primary antibodies: anti-GAPDH (1:5000), anti-E-cad-
herin (1:2000), and anti-α-SMA (1:2000) at 4°C overnight. The membranes were then incubated with the secondary antibody (1:1000). The results were analyzed by image analysis software, and the gray values of the protein electrophoresis strip were calculated.

**Statistical analysis**

The experimental data were analyzed by SPSS software 16.0 (SPSS Inc., USA). The approximate normal distribution of the experimental data was presented as mean ± standard deviation (SD). The comparison between the different groups was calculated using one-way analysis of variance, and the correlation between variables was calculated using Pearson correlation analysis. A P < 0.05 was considered statistically significant.

**Results**

**LPS induces EMT in HPMCs cells**

To confirm if LPS induced EMT, we detected the expressions of E-cadherin and α-SMA in a human peritoneal mesothelial cell line, HPMCs were cultured with LPS. To determine the dose of LPS for all subsequent experiments, HPMCs were cultured in media containing several concentrations of LPS. Based on the MTT assay, we found that 0 to 20 μg/mL of LPS did not change the cell viability of the HPMCs [Figure 1A]. We chose 10 μg/mL of LPS based on a previous study. To investigate the effects of matrine on LPS-induced EMT in HPMCs, we wanted to determine the appropriate dose of matrine to be used in the MTT experiments. We found that 0 to 2 g/L of matrine did not influence the cell viability of HPMCs. We tested different concentrations of matrine: 0.2 g/L (low dose), 0.4 g/L (medium dose), and 0.6 g/L (high dose).

The HPMC cells were treated with 10 μg/mL of LPS for 24h in the absence or presence of matrine. The wound healing assay revealed that 0.4 g/L of matrine reduced the migratory ability of the HPMCs induced by LPS and found that 10 μg/mL of LPS increased the migratory ability of the HPMCs [Figure 2].

The effects of matrine on LPS-induced EMT of HPMCs were further assessed using RT-PCR and Western blot analyses. LPS-induced EMT was alleviated by co-treating the cells with matrine. These results were validated by the observed reduced upregulation of α-SMA [Figure 3B] and amelioration of the inhibited expression of E-cadherin at the mRNA [Figure 3A] and protein [Figure 4] levels. Furthermore, these effects were dose-dependent. These findings suggested that matrine attenuates LPS-induced EMT in HPMCs.

**Matrine ameliorates the LPS-induced inhibition of miR-29b and miR-129-5p**

MicroRNAs, miR-29b and miR-129-5p are small RNAs that are closely associated with fibrosis. By RT-PCR, we found that 10 μg/mL of LPS inhibited the expression levels of miR-29b and miR-129-5p in HPMCs. In contrast, when matrine was added, the expression levels of miR-29b and miR-129-5p increased, in a dose-dependent manner [Figure 5]. Taken together, these findings implicated that the recovery of miR-29b and miR-129-5p is an important event in the matrine-induced anti-EMT effect observed in HPMCs.

In conclusion, it is clear that PD is becoming more widespread. A previous report by Higashi et al suggested that cell polarity weakens with increased duration of PD, peritoneal dialysis effluent-derived cells (PDE-HPMC) expressed the EMT marker, α-SMA. Thus, preventing and treating this early and reversible phase of peritoneal fibrosis remains a popular focus of current research.

LPS promotes EMT, as was observed by work from other groups, where the expression of E-cadherin was significantly decreased and the expressions of collagen I, vimentin, α-SMA were increased, when HPMC were treated with LPS to simulate chronic inflammation. We successfully reproduced the EMT model of LPS-stimulated HPMC, in our study, as was validated by the observed decrease in E-cadherin and increase in α-SMA expression levels.

**Figure 1:** The effects of LPS, matrine on HPMCs cell viability (MTT). (A) HPMCs were exposed to different concentrations of LPS (0.5, 1.0, 2.0, 5.0, 10.0, 20.0 μg/mL) for 72h the absorbance value of the sample was measured, there were no significant differences in different concentration of LPS group and blank control group (P > 0.05). (B) HPMCs were cultured in media containing several concentrations of matrine (0.1, 0.2, 0.5, 1.0, 1.5, 2.0 g/L) for 72h, the absorbance value of the sample was measured. There were no significant differences in different concentration of LPS group and blank control group (P > 0.05). HPMCs: Human peritoneal mesothelial cells; LPS: Lipopolysaccharide; Mat: Matrine.
Figure 2: The changes of cell migration ability of different drugs stimulated HPMCs. After scratch, the cell gap of the LPS group was smaller than that of the control group and LPS + matrine group in 8 h; the notch of the scratch of LPS group gradually decreased in 16 h, while the gap of scratch in the control group and LPS + Mat group was still wide. After 24 h, the gap of LPS group disappeared, but there were still obvious gaps of the scratch between the control group and LPS + Mat group. HPMCs: Human peritoneal mesothelial cells; LPS: Lipopolysaccharide; Mat: Matrine.

Figure 3: The mRNA expression of E-cadherin and \(\alpha\)-SMA after LPS induction and treating with different concentrations of matrine in HPMCs. HPMCs incubated with DMEM, LPS, LPS + 0.2 g/L Mat, LPS + 0.4 g/L Mat, LPS + 0.6 g/L Mat for 24 h. The mRNA expression levels of E-cadherin (A) and \(\alpha\)-SMA (B) were detected using reverse transcription-polymerase chain reaction analysis. All data are presented as the mean \(\pm\) standard deviation. GAPDH was used as an internal control (* \(P < 0.05\) vs. LPS group, † \(P < 0.05\) vs. control group). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HPMCs: Human peritoneal mesothelial cells; LPS: Lipopolysaccharide; Mat: Matrine; \(\alpha\)-SMA: Alpha-smooth muscle actin.
Figure 4: The expression of E-cadherin and α-SMA after LPS induction and treating with different concentrations of matrine in HPMCs. Expression of E-cadherin, α-SMA in HPMCs after incubation with DMEM, LPS, LPS + 0.2 g/L Mat, LPS + 0.4 g/L Mat, LPS + 0.6 g/L Mat for 72 h, and the expression levels of E-cadherin and α-SMA were detected using Western blot analysis (A). All data are presented as the mean ± standard deviation (B, C). GAPDH protein was used as an internal control (∗P < 0.05 vs. LPS group, †P < 0.05 vs. control group), DMEM: Dulbecco’s modified Eagle’s medium; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HPMCs: Human peritoneal mesothelial cells; LPS: Lipopolysaccharide; Mat: Matrine; α-SMA: Alpha-smooth muscle actin.

Figure 5: The expression of miR-29b and miR-129-5p after LPS induction and treating with different concentrations of matrine in HPMCs. HPMCs incubated with DMEM, LPS, LPS + 0.2 g/L Mat, LPS + 0.4 g/L Mat, LPS + 0.6 g/L Mat for 24 h. The mRNA expression levels of miR-29b (A) and miR-129-5p (B) were detected using reverse transcription-polymerase chain reaction analysis. All data are presented as the mean ± standard deviation. GAPDH was used as an internal control (∗P < 0.05 vs. LPS group, †P < 0.05 vs. control group), DMEM: Dulbecco’s modified Eagle’s medium; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HPMCs: Human peritoneal mesothelial cells; LPS: Lipopolysaccharide; Mat: Matrine; α-SMA: Alpha-smooth muscle actin.
A previous study found that matrine alleviated LPS-stimulated intestinal inflammation and oxidative stress by down-regulating IL-1β, IL-17 and malondialdehyde. In addition, matrine was shown to prevent LPS-induced acute lung injury by decreasing the expressions of COX-2 and ICAM-1 and reducing the production of IL-6, IL-8, MCP-1, and CCL5. Moreover, matrine inhibited EMT in human brain glioma cells by acting on the p38MAPK and AKT signaling pathways, increasing the expression of the epithelial cell marker, E-cadherin, and reducing the expression of the mesenchymal cell marker, N-cadherin.

In our study, we wanted to determine if matrine could inhibit LPS-induced EMT. We used the MTT assay and found that about 0.1 to 2.0 g/L of matrine did not affect the growth of HPMC. Next, we used the wound scratch assay and found that matrine inhibited the LPS-induced enhanced migratory ability of HPMC. RT-PCR and Western blots further confirmed that matrine inhibits LPS-induced EMT in HPMC.

Peritoneal fibrosis is associated with several miRNAs, like miR-29b and miR-129-5p. Treatment with targeted miRNAs can effectively reverse EMT and inhibit fibrosis. A study found that when murine peritoneal function is impaired, the expression levels of miR-29b in peritoneal mesothelial cells decreased. Moreover, when miR-29b was overexpressed, E-cadherin expression levels increased and α-SMA and collagen I expression levels decreased, strongly suggesting that miR-29b inhibits EMT to prevent peritoneal dysfunction. In addition, it was speculated that blocking the Sp1-TGF-β/Smad3 pathway could be the reason why miR-29 b inhibits EMT.

Another study found that miR-129-5p inhibited EMT via PDPK1. SIP1 interacts with Smad proteins to inhibit the TGF-β pathway and overexpress matrix metalloproteinases. SIP1 also inhibits the activity of the E-cadherin promoter and enhances the activity of the vimentin promoter.

In conclusion, the advantage of this research was that we studied them at the cellular level, we found that matrine reversed LPS-induced EMT. Matrine can alleviate the inhibition of E-cadherin expression and reduce the expression of α-SMA in LPS-induced HMPCs. In addition, we found that LPS inhibited the expressions of miR-29b and miR-129-5p in HMPCs, while matrine elevated the expressions of miR-29b and miR-129-5p. Given that matrine exhibits antibacterial, anti-inflammatory properties, our data suggest that it can be used as a potential drug in the treatment of Peritoneal dialysis associated peritonitis (PDA) and peritoneal fibrosis. Next, we will establish an uremia model in animals to study the effect of matrine on peritoneal fibrosis induced by peritonitis.

Conflicts of interest
None.

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How to cite this article: Li YZ, Peng X, Ma YH, Li FJ, Liao YH. Matrine suppresses lipopolysaccharide-induced fibrosis in human peritoneal mesothelial cells by inhibiting the epithelial-mesenchymal transition. Chin Med J 2019;132:664–670. doi: 10.1097/CM9.0000000000001227