Matrine inhibits production and actions of fibrogenic cytokines released by mouse peritoneal macrophages[1]

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KEY WORDS matrine; macrophages; liver cirrhosis; cell division; collagen; transforming growth factor beta

ABSTRACT

AIM: To study the effects of matrine (Mat) on production and actions of fibrogenic cytokines from mouse peritoneal macrophages. **METHODS**: Mouse peritoneal macrophages were primed with calcimycin 1 μmol/L for 8 h then elicited by lipopolysaccharides (LPS) 100 μg/L for 6 h to induce fibrogenic cytokines. Proliferative and collagen stimulating activity in the macrophage culture supernatants was determined by crystal violet staining assay and [^{3}H]-proline incorporation assay using rat hepatic stellate HSC-T6 cell or mouse fibroblast NIH3T3 cell. Transforming growth factor β (TGFβ) activity was measured by [^{3}H]-thymidine incorporation assay using Mv-1-Lu mink lung epithelial cell. **RESULTS**: Mat (0.5 - 2 mmol/L) was shown to significantly inhibit LPS-induced collagen stimulating activities and TGFβ production ($P < 0.01$) whereas did not inhibit proliferative activities induced by macrophages. Macrophage conditioned medium (MCM)-driven proliferation and collagen synthesis of HSC-T6 cells as well as NIH3T3 cells were attenuated by Mat (0.5 - 2 mmol/L) in a concentration-dependent manner. **CONCLUSION**: Antifibrotic effects of Mat on hepatic stellate cells may be related to reduction of fibrogenic cytokine production and blockade of their actions.

INTRODUCTION

Liver fibrosis is characterized by two major events: proliferation of hepatic stellate cells (HSC) and increased synthesis of extracellular matrix (ECM), particularly collagen. It is a common pathophysiological process of chronic liver diseases to liver cirrhosis. Monocytes/macrophages have been postulated to play an important role in the development of fibrosis of liver that was injured by viral infection, alcohol, and various drugs. Fibrotic liver injuries result in activation of macrophages and release of fibrogenic cytokines, such as platelet derived growth factor (PDGF), transforming growth factor beta-1 ($TGF\beta_1$), and many stimulating factors, which activated HSC, stimulated ECM synthesis, and diminished matrix degradation that resulted in a net matrix accumulation. This constitutes the final common pathway in all forms of liver fibrosis[1,2]. Therefore, manipulation of these cytokines may provide a novel therapeutic avenue for treatment of this disease either by reducing endogenous cytokine production or blocking the actions of these fibrogenic cytokines.

Matrine (Mat), an alkaloid found in species of *Sophra* plants in *Leguminosae*, has been used in treatment of chronic liver disease. It has been shown that Mat reduced liver fibrosis in a rat hepatotoxic model[3,4], but the mechanisms of its action were still unknown. The present report described the effect of Mat on the fibrogenic cytokine production by mouse peritoneal macrophages, and action of macrophage conditioned medium (MCM)-driven proliferation and collagen synthesis of HSC-T6 cells[5] and NIH3T3 cells.

MATERIALS AND METHODS

Reagents Mat (mp 75.5 - 77.5 ℃, purity > 99 %) was obtained from Yanchi Pharmaceutical Factory, Ningxia, China. Lipopolysaccharides (LPS, *E coli* 0111 : B4) and calcimycin were from Sigma (St Louis, USA). Dulbecco's modified Eagle's medium (DMEM) was from Gibco BRL (Karlsruhe, Germany). [^{3}H]-Proline and [^{3}H]-thymidine were purchased from

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China Institute of Atomic Energy and Shanghai Institute of Nuclear Research, Chinese Academy of Sciences.

Animals and cell lines ICR mice, ♀, weighing (28±3) g, were from the Animal Center of Second Military Medical University (Grade Ⅱ, Certificate № 28-48).

HSC-T6 cell, an immortalized rat myofibroblast line, which had the stable phenotype and biochemical characters, was kindly provided by Dr SL Friedman (Liver Center Laboratory, San Francisco General Hospital, USA). NIH3T3 fibroblast and Mv-1-Lu mink lung epithelial cells were from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. All the cells were cultured in DMEM with 10 % calf serum at 37 ℃ in a humidified atmosphere of 5 % CO_2 + 95 % air.

Induction of fibrogenic cytokines from macrophages Peritoneal exudate cells (PEC) were obtained from thioglycollate-injected ICR mice. The cell suspension was adjusted to 2×10^9/L in DMEM containing 10 % calf serum and calcimycin 1 μmol/L, and dispensed at 1 mL per well in 24-well microplate. After 8-h incubation, the cells were washed with culture medium, and stimulated with LPS 100 μg/L in the presence or absence of Mat for 6 h to induce fibrogenic cytokines. The supernatants were collected and assayed for the mitogenic and collagen stimulating activity in HSC-T6 cells.

The macrophage conditioned medium (MCM) was prepared as described below. The cells were primed with calcimycin and elicited by LPS, then washed with PBS three times and incubated with DMEM for 24 h. The suspernatants were pooled and stored until use.

Cell proliferation Proliferative activity in macrophage culture supernatants was evaluated by crystal violet assay[6]. HSC-T6 cells (1×10^4 per well) were seeded in 96-well microplate and incubated for 24 h. Serial diluted supernatants were added to the wells. After a 48-h incubation, cell density was measured and expressed as A_{595} at 1:8 dilution.

To test the effect of Mat on MCM-driven proliferation of HSC-T6 cells or NIH3T3 cells, the culture medium was replaced with DMEM supplemented with 0.4 % calf serum and incubated for 48 h. Mat and MCM (1:4, v/v) were then added simultaneously and incubated for another 24 h.

Collagen synthesis Collagen stimulating activity was measured by [^{3}H]-proline incorporation assay[6]. HSC-T6 cells (3.5×10^4 per well) were incubated in DMEM with 10 % calf serum, ascorbic acid 50 mg/L, together with the serial diluted supernatants for 24 h, and labeled with [^{3}H]-proline 7.4 kBq per well for an additional 24 h. The cells were harvested and the radioactivity was counted in a liquid scintillation counter. Collagen stimulating activity was expressed as Bq at 1:8 dilution.

To test the effect of Mat on MCM-driven collagen synthesis of HSC-T6 cells or NIH3T3 cells, the culture medium was replaced with DMEM supplemented with 2 % calf serum, MCM (1:4, v/v) and ascorbic acid 50 mg/L with or without Mat.

Bioassay for transforming growth factor β TGFβ activity was monitored by Mv-1-Lu target cell proliferative inhibition assay[7]. Mv-1-Lu cells (2.5×10^4 per well) were seeded and incubated in 96-well microplate for 24 h, then incubated with sample dilution series for 36 h. To assess cell proliferation, [^{3}H]-thymidine 7.4 kBq per well was added and incubated for a further 12 h. The cells were then harvested onto glass fibers and the radioactivity incorporated into DNA was measured using a scintillation counter. TGFβ activity was expressed as Bq at 1:8 dilution.

Statistics Data were analyzed by ANOVA and t-test, and expressed as $\bar{x} \pm s$.

RESULTS

Effects of matrine on proliferative activity, collagen stimulating activity, and TGFβ production by macrophages Exposure of mouse peritoneal macrophages to Mat (0.5 - 2 mmol/L) markedly inhibited LPS-induced collagen stimulating activities and TGFβ production in a concentration-dependent manner, but Mat at all concentrations studied did not affect proliferative activities induced by macrophage (Tab 1).

Effect of matrine on proliferation of MCM-stimulated HSC-T6 cells and NIH3T3 cells The macrophage-conditioned medium (1 : 4) markedly enhanced proliferation of rat hepatic stellate HSC-T6 cells and mouse fibroblast NIH3T3 cells, with a proliferation rate of 39.2 % and 86.0 % respectively ($P < 0.01$). Mat significantly attenuated MCM-driven HSC-T6 cell proliferation at the concentration of 1 and 2 mmol/L ($P < 0.01$), while exposure of NIH3T3 cells to Mat resulted in a concentration-dependent proliferative suppression (Tab 2).

Effect of matrine on collagen synthesis in MCM-stimulated HSC-T6 cells and NIH3T3 cells MCM (1:4) significantly induced collagen synthesis of HSC-T6 cells and NIH3T3 cells, with an increase rate of

Tab 1. Effects of matrine on LPS-induced proliferative activity, collagen stimulating activity, and TGFβ production by mouse peritoneal macrophages primed with calcimycin. Proliferative activity and collagen stimulating activity were assessed as crystal violet and [^{3}H]-proline uptake by HSC-T6 cells, and TGFβ activity as inhibition of [^{3}H]-thymidine uptake by Mv-1-Lu cells. $n=4$. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ vs matrine 0 mmol·L^{-1}.

Matrine/ mmol·L^{-1}	A_{595}	Collagen stimulating activity/Bq	TGFβ activity/ Bq
0	1.24±0.06	264±13	19±4
0.5	1.20±0.03^a	200±10^c	23±2.9^a
1.0	1.19±0.03^a	185±9^c	26.4±2.6^b
2.0	1.24±0.01^a	163±6^c	35±3^c

Tab 2. Effect of matrine on macrophage conditioned medium (MCM)-driven proliferation of rat hepatic stellate HSC-T6 cells and mouse fibroblast NIH3T3 cells. Proliferation was assessed by crystal violet assay and expressed as A_{595}. $n=6$ wells. $\bar{x} \pm s$. $^aP>0.05$, $^cP<0.01$ vs control (without matrine).

Matrine/ mmol·L^{-1}	A_{595} HSC-T6	A_{595} NIH3T3
Medium	0.74±0.01	0.50±0.05
Control	1.03±0.09	0.93±0.01
0.25	1.01±0.09^a	0.94±0.10^a
0.50	0.97±0.09^a	0.84±0.05^c
1.00	0.91±0.10^c	0.79±0.08^c
2.00	0.82±0.10^c	0.56±0.04^c

106.1 % and 72.6 % respectively ($P<0.01$). Mat reduced the MCM-driven collagen synthesis in a concentration-dependent manner. Mat (2 mmol/L) totally blocked the collagen stimulating effect of MCM (Tab 3).

Tab 3. Effect of matrine on MCM-driven collagen synthesis of HSC-T6 cells and NIH3T3 cells. Collagen synthesis was assessed as [^{3}H]-proline uptake. $n=6$. $\bar{x} \pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ vs control (without matrine).

Matrine/ mmol·L^{-1}	[^{3}H]-proline uptake/Bq HSC-T6	[^{3}H]-proline uptake/Bq NIH3T3
Medium	169±71	174±21
Control	348±68	300±25
0.25	357±62^a	313±28^a
0.50	268±38^b	240±13^b
1.00	224±55^c	185±14^c
2.00	151±5^c	155±6^c

DISCUSSION

Cytokines synthesized and secreted by macrophages have been shown to be involved in the pathogenesis of hepatic fibrosis. They exert different effects on hepatic stellate cells. For example, PDGF, tumor necrosis factor α, and interleukin-1α appear to stimulate HSC proliferation, whereas TGFβ_1 may inhibit HSC proliferation but potentiate the stimulatory effect of PDGF. In addition, TGFβ_1 primarily enhances the synthesis of extracellaluar matrix[8-10]. Therefore, we focused on macrophage conditioned medium as a stimulating factor to study the *in vitro* antifibrotic effect of Mat. Our results demonstrated that under the present experimental conditions, exposure of HSC-T6 cells to MCM resulted in enhancement in both proliferative activity and collagen synthesis capacity, with an increase rate of 39.2 % and 106.1 % respectively. These were similar to those obtained with cultured rat hepatic lipocytes and Kupffer cell conditioned medium[11]. Exposure of fibroblast NIH3T3 cells to MCM had a greater effect on proliferation (86.0 %) and weaker effect on collagen synthesis (72.6 %) compared to the results obtained from HSC-T6 cells. These different reactions may contribute to the different physiological properties of myofibroblast and fibroblast. Treatment with Mat decreased MCM-driven proliferation and collagen synthesis of HSC-T6 cells and NIH3T3 cells in a concentration-dependent manner. Monocyte-conditioned medium from monocytes of patients with liver disease is fibroproliferative and this fibroproliferation can be blocked by PDGF antibody[12]. PDGF accounts for 50 % - 70 % of the total macrophage-derived mitogenic activity and TGFβ_1 is primarily responsible of collagen synthesis. Mat completely prevents PDGF- and TGFβ_1-stimulated proliferation and collagen synthesis at a high concentration[4]. These effect may partially explain the blocking effect of Mat on the macrophage and hepatic stellate cell communication.

Mat has been shown to inhibit TNFβ, IL-1, and IL-6 production *in vitro* and *in vivo*[13,14]. In the present study, treatment of macrophages with Mat diminished the collagen stimulating activity and TGFβ released by macrophage. These results indicated that Mat could suppress the fibrogenic cytokine production.

In summary, the present study suggested that the inhibitory effects on the fibrogenic cytokine production and actions by matrine might be one of the important aspects of its antifibrotic mechanisms.

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苦参碱抑制小鼠腹膜巨噬细胞纤维化细胞因子的产生和作用[1]

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关键词 苦参碱; 巨噬细胞; 肝硬化; 细胞分裂; 胶原; 转化生长因子 β

目的: 研究苦参碱对小鼠腹腔巨噬细胞释放纤维化细胞因子以及对巨噬细胞条件培养基(MCM)促HSC-T6大鼠储脂细胞和NIH3T3成纤维细胞增殖和胶原合成的影响. **方法**: 巨噬细胞先后用卡西霉素1 μmol/L和脂多糖100 μg/L刺激诱导产生纤维化因子. 细胞上清中促细胞增殖活性和促胶原合成活性分别用结晶紫染色法和[^{3}H]-脯氨酸掺入法测定. 转化生长因子β活性采用貂肺上皮Mv-1-Lu细胞增殖抑制法测定. **结果**: 苦参碱(0.5 – 2 mmol/L)显著抑制LPS诱导的促胶原合成活性和TGFβ的产生, 但不能抑制巨噬细胞产生促细胞增殖活性; 苦参碱还能剂量依赖地抑制MCM诱导的HSC-T6细胞以及NIH3T3细胞增殖和胶原合成. **结论**: 苦参碱抗肝纤维化作用与抑制巨噬细胞纤维化因子的产生和阻断其作用有关.

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