Effects of lipopolysaccharides stimulated Kupffer cells on activation of rat hepatic stellate cells

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Received: 2003-07-04 Accepted: 2003-09-01

ABSTRACT
AIM: To study the effects of Kupffer cell-conditioned medium (KCCM) derived from lipopolysaccharide (LPS) treatment on proliferation of rat hepatic stellate cells (HSC).

METHODS: HSC and Kupffer cells were isolated from the liver of Wistar rats by in situ perfusion with pronase and collagenase and density gradient centrifugation with Nycodenz and cultured. KCCM was prepared and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to detect HSC proliferation. The content of type IV collagen and laminin secreted by HSC in the HSC-conditioned medium was determined by radioimmunoassay. TGF-β1 production in the KCCM was detected by enzyme-linked immunosorbent assay (ELISA).

RESULTS: HSC and Kupffer cells isolated had high purity. One microgram per milliliter LPS-activated KCCM and unstimulated KCCM could significantly promote HSC proliferation [0.132±0.005 and 0.123±0.008 vs control group (0.100±0.003), P<0.01], and there was a difference between them (P<0.05). Ten microgram per milliliter LPS-activated KCCM (0.106±0.030) was unable to promote HSC proliferation (P>0.05). Adding anti-TGF-β1 antibodies could suppress the proliferation promoted by unstimulated KCCM and LPS (1 µg/ml)-activated KCCM (0.109±0.009 vs 0.123±0.008, 0.115±0.008 vs 0.132±0.005, P<0.01). LPS (1 µg/ml or 10 µg/ml) could not promote HSC proliferation immediately (0.096±0.003 and 0.101±0.004 vs 0.100±0.003, P>0.05). There was a parallel behavior between HSC proliferation and increased ECM level. One microgram per milliliter LPS-activated KCCM contained a larger amount of TGF-β1 than unstimulated KCCM.

CONCLUSION: The technique for isolation of HSC and Kupffer cells described here is simple and reliable. KCCM stimulated by LPS may promote HSC proliferation and collagen accumulation, which are associated with hepatic fibrogenesis.

Zhang X, Yu WP, Gao L, Wei KB, Ju JL, Xu JZ. Effects of lipopolysaccharides stimulated Kupffer cells on activation of rat hepatic stellate cells. World J Gastroenterol 2004; 10 (4): 610-613
http://www.wjgnet.com/1007-9327/10/610.asp

INTRODUCTION
Hepatic fibrosis is now regarded as a common response to chronic liver injury. Regardless of its nature (viral infections, alcohol abuse, and metal overload), it is also characterized by excessive deposition of extracellular matrix components[1-3]. Hepatic stellate cells (HSC) are a major source of extracellular matrix in normal and pathological conditions[4,6]. During fibrogenesis, HSC undergoes a process of activation, developing a myofibroblast-like phenotype associated with increased proliferation[7,8] and collagen synthesis[10,11]. As the data suggest, a paracrine mechanism may prevail over the stimulation of HSC proliferation and collagen synthesis in vivo[12,13]. Lipopolysaccharide (LPS) is a critical component of the cell membrane of gram-negative bacteria and can mediate pathophysiological alterations during endotoxaemia. Systemic exposure to LPS could result in a cascade of events involving cellular and soluble mediators of inflammation, leading to injury to organs, including liver[14,15]. Because LPS is known to activate Kupffer cells to release various kinds of cytokines[16,17], there may be a chain of events to cause hepatic fibrosis.

The aim of this study was to evaluate whether paracrine stimuli derived from LPS-activated Kupffer cells could induce HSC proliferation and collagen synthesis or LPS could stimulate HSC proliferation immediately.

MATERIALS AND METHODS

Materials
Pronase E, collagenase type IV, deoxyribonuclease I, MTT, Nycodenz [5-N-2, 3-dihydroxypropylaceta-mido]-2, 4, 6-tri-iodo-N, N’ bis -(2, 3-dihydroxypropyl) isophthalamide] and lipopolysaccharide (LPS, Escherichia coli, serotype O111: B4) were obtained from Sigma. All immunocytochemical reagents, including rabbit antihuman desmin and rabbit antihuman lysozyme, were purchased from Maixin Co. (Fuzhou, China). Culture media, Dulbecco’s modified Eagle’s medium (DMEM) and PRMI1640 were purchased from GIBCO (Grand Island, NY). Newborn bovine serum and fetal bovine serum were purchased from Sijiqing Co. (Hangzhou, China).

Methods
HSC and Kupffer cell isolation and culture[19-22] HSC and Kupffer cells were isolated from rat livers by the pronase-collagenase method. Briefly, after the rat (male Wistar rats ranging from 350 g to 450 g of body weight) was anesthetized with sodium pentobarbital (40 mg/kg body weight, ip), the abdomen was opened and the portal vein was cannulated with 16-gauge cannula. The liver was perfused in situ for 15 min with Ca2+/Mg2+-free Hank’s balanced salt solution (pH 7.4, 37°C) at a flow rate of 15 mL/min, and was then perfused with Hank’s balanced salt solution (pH 7.4, 37°C) containing 0.1% pronase E and 0.05% collagenase type IV for an additional 30 min at a flow rate of 30 mL/min. After perfusion, the liver was removed, cut into small pieces, and incubated in a bath of Hank’s balanced salt solution containing 0.02% pronase E and 0.05% collagenase type IV and 0.001% deoxyribonuclease at 37°C for 20 min. After passing through a filter (mesh size 74 µm), cells were washed twice with Hank’s balanced salt

BRIEF REPORTS

World J Gastroenterol 2004;10(4):610-613
World Journal of Gastroenterology
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solution and HSC were obtained by centrifugation over 12% (wt/vol) Nycoden gradient for 18 min at 1 450 g. After centrifugation, HSC were collected from the interface, washed with Hank’s balanced salt solution, and resuspended at a concentration of 1×10^6 cells/ml, in DMEM supplemented with 20% newborn bovine serum, 2 mmol/L glutamine, and 1% antibiotic solution. Kupffer cells were synchronously collected under the interface, washed with Hank’s balanced salt solution, and resuspended at a concentration of 1×10^6 cells/ml, in PRMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and 1% antibiotic solution. Cells were then cultured at 37°C in a humidified atmosphere containing 5% CO₂. The media were removed 4 h later until use.

Preparation of Kupffer cell-conditioned medium (KCCM)

After a 72 h recovery period from isolation, the primary Kupffer cells were cultured in the presence or absence of 1 µg/ml or 10 µg/ml of LPS. Conditioned medium was collected after 48 h of culture and filtered with a 0.45 µm membrane filter, and stored at -70°C until use.

Modulation of HSC proliferation by KCCM or LPS

HSC were placed in 96-well plates and cultured for 48 h in the presence or absence of KCCM or LPS (1 µg/ml or 10 µg/ml). The HSC, with addition of KCCM, were cultured in the presence or absence of rabbit anti-human TGF-β₁, immunoglobulin. Conditioned media were collected and stored at -70°C until use. The remaining cells were added with 180 µl media and 20 µl MTT (5 g/L) at 37°C. The media were removed 4 h later and formazan product was dissolved with 100 µl/well of dimethyl sulphoxide (DMSO). After a few minutes at room temperature, to ensure that all crystals were dissolved, the absorbance at 570 nm and referent wavelength of 630 nm was determined by trypan blue exclusion test.

Modulation of collagen type IV and laminin secretion by KCCM or LPS

The amount of type IV collagen and laminin, the most abundant type of ECM in early liver fibrosis, secreted by HSC in the HSC-conditioned medium was measured by radioimmunoassay.

Characterization of stimulatory factor in LPS-activated KCCM

TGF-β₁ production in the culture supernatant was measured by ELISA.

Statistical analysis

Data were expressed as mean±SD. Differences between any two groups were determined by new Duncan test and t-test. P<0.05 was considered statistically significant.

RESULTS

Isolation and culture of HSC and Kupffer cells

The HSC isolated with the aforementioned Nycoden cushion ranged from 5×10^6 to 1×10^7 cells per liver, and Kupffer cell ranged from 3×10^6 to 5×10^6 cells per liver. The cell viability determined by trypan blue exclusion test varied considerably, but was usually higher than 80%. HSC were identified by their typical dark-light microscopic appearance due to abundant vitamin A-containing vacuoles which showed intense fluorescence upon irradiation at 328 nm, and by positive immunocytochemical staining for desmin. Kupffer cells were identified by positive immunocytochemical staining for lysozyme. The initially isolated Kupffer cells were spherules with refractation which were much smaller than hepatocytes and HSC. Kupffer cells adhered to plates and some spread their pseudopods 4 h after seeding. During the first 24 h of HSC culture, the cells rounded up and flattened out. The major fraction contaminating nonadherent lymphocytes and endothelial cells, was removed with the first change of medium. After 2 to 3 d in culture, HSC developed a characteristic stellate shape. Lipid droplets had visible round nuclei and decreased with the time of culture. Confluence was reached on about the 14th day.

Effect of KCCM or LPS on HSC proliferation

When cells were cultured in the presence of 1 µg/ml or 10 µg/ml LPS, HSC proliferation was not observed (P>0.05). When cells were cultured in the presence of unstimulated KCCM and 1 µg/ml LPS-activated KCCM, HSC proliferation remarkably (P<0.01), and there was a difference between the two groups. Ten µg/ml LPS-activated KCCM could not promote HSC proliferation (P>0.05). In addition, HSC proliferation promoted by unstimulated KCCM and 1 µg/ml LPS-activated KCCM was suppressed partly in the presence of anti-TGF-β₁ antibodies (P<0.01), but there was a difference compared with control group (P<0.01) (Table 1).

Effect of KCCM or LPS on collagen type IV and laminin secretion by HSC

When HSC were cultured in the presence of 1 µg/ml or 10 µg/ml LPS, the amount of collagen type IV secreted by HSC was 29.17±1.36 µg/L and 50.33±0.97 µg/L, respectively, and there was no difference between them (P>0.05). The amount of laminin was 56.61±2.39 ng/ml and 57.12±2.00 ng/ml respectively without statistical difference (P>0.05). When HSC were cultured in the presence of unstimulated KCCM and 1 µg/ml LPS-activated KCCM, collagen type IV and laminin secreted by HSC were markedly enhanced by LPS-stimulated KCCM (42.39±1.74 µg/L and 69.40±1.86 ng/ml), but were only slightly enhanced by unstimulated KCCM (37.99±1.41 µg/L and 63.54±2.25 ng/ml). Furthermore, this enhanced secretion was partly blocked in the presence of anti-TGF-β₁ antibodies. Ten microgram per milliter LPS-activated KCCM could not promote collagen type IV and laminin secretion (31.12±0.84 µg/L and 56.34±2.17 ng/ml, P>0.05) (Table 2).
with chronic liver disease, such as hepatic cirrhosis and were conflicting. The role of Kupffer cells in stimulating HSC, although the results have shown that the functions of adjacent cells were influenced markedly higher (0.941±0.114) than that in unstimulated KCCM (0.465±0.075).

Control group 30.34±1.02 57.56±1.56
KCCM1(unstimulated) 0.465±0.075
KCCM2(1 µg/ml LPS-activated) 0.669±0.093
KCCM3(10 µg/ml LPS-activated) 36.26±0.91
KCCM1+anti-TGF-β1 antibodies 30.76±1.88 56.16±2.62
Control group 30.34±1.02 57.56±1.56

**DISCUSSION**

It has been recognized that HSC are the major source of extracellular matrix in normal and pathological conditions[1,5,9]. HSC activation during fibrogenesis, as a result of injury, is a dynamic process, which leads from a quiescent state to a myofibroblast-like phenotype with increased production of extracellular matrix and enhanced proliferation. Therefore, HSC play a central role in hepatic fibrogenesis. Previous studies have shown that the functions of adjacent cells were influenced by mediators released from Kupffer cells, such as protein synthesis in hepatocytes[27] and sinusoidal endothelial cell functions[28]. Several investigators have already evaluated the role of Kupffer cells in stimulating HSC, although the results were conflicting[29]. Endotoxemia has been found in patients with chronic liver disease, such as hepatic cirrhosis and hepatitis[30,31]. Endotoxemia and hepatocytic damage could affect each other, but there is no evidence to show whether endotoxemia could promote HSC activation. So the relation between LPS and HSC was studied to demonstrate the possible mechanism in the fibrogenesis of endotoxemia.

We established an in vitro model of HSC and Kupffer cell-conditioned medium. The primary HSC grew very slowly and confluence was reached on about the 14th day in culture. The subcultured HSC grew much faster than the primary HSC, and lipid droplets decreased and even disappeared, which accorded with previous studies. In this study, Kupffer cells and HSC were cultured with LPS (1 µg/ml or 10 µg/ml), and Kupffer cell-conditioned medium was cultured with HSC. This mimicked the process that intestinal LPS affected liver cells in vivo when endotoxemia occurred. We measured HSC proliferation after HSC were cultured with LPS directly or indirectly to evaluate the degree of HSC activation. Our data showed that unstimulated KCCM could promote HSC proliferation. When HSC were cultured in the presence of 1 µg/ml LPS-activated KCCM, HSC proliferated more remarkably than the former. It was suggested that the primary Kupffer cells could secret some factors and LPS could stimulate synthesis and secretion of these factors by Kupffer cells. When HSC were cultured in the presence of LPS directly, HSC proliferation could not be observed. Our results indicated that LPS affected HSC indirectly. Ten µg/ml LPS-activated KCCM could not promote HSC proliferation. It is difficult to assess and explain the reason why high concentration LPS could not activate HSC. One possibility was that high concentration LPS might decrease or suppress Kupffer cells to secrete cytokines, which could stimulate HSC proliferation. The other possibility was that cytokines secreted by Kupffer cells did not decrease, but Kupffer cells could secrete some other cytokines and counteract the promotion. So HSC did not undergo proliferation.

Overload ECM synthesis could lead to hepatic fibrosis. In our study, we measured the relative concentration of collagen type IV and laminin in HSC-conditioned media. Our results showed that there was a parallel behavior between increased HSC proliferation and increased ECM level. It was suggested that HSC proliferation was the direct reason of the overload ECM synthesis, which was probably related with the increased cell number and capability of single HSC to secret ECM.

TGF-β1 is the most powerful cytokine to promote fibrosis. During the period of HSC activation, TGF-β1 was a very important initiation factor[32]. In our study, HSC proliferation and ECM production by unstimulated KCCM and 1 µg/ml LPS-activated KCCM were suppressed partly in the presence of anti-TGF-β1 antibodies, suggesting that HSC proliferation might be related with TGF-β1 and some other cytokines, because anti-TGF-β1 antibodies could completely not suppress the effect of proliferation. Our results suggested that Kupffer cells could be activated by a moderate concentration of LPS and probably release some soluble mediators, such as TGF-β1, presented in the LPS-activated Kupffer cell-conditioned medium, which activated HSC from a quiescent state to a myofibroblast-like phenotype. It is clear that the stimulatory effect on both HSC proliferation and collagen synthesis is the main feature of liver fibrosis. It is easy to measure cytokines contained in LPS-activated KCCM by ELISA. Our results showed that moderate concentration of LPS-activated KCCM contained larger-activated KCCM and approve the above deducibility, i.e., a high concentration of LPS might promote Kupffer cells to secrete a smaller amount of TGF-β1 than 1 µg/ml LPS, and secret other cytokines to counteract the effect of TGF-β1.

In conclusion, LPS plays a role in the stimulation of HSC proliferation and collagen synthesis. Kupffer cells cultured in

**Table 2** Effect of KCCM or LPS on collagen type IV and laminin secretion by HSC (mean±SD, n=6)

| Treatment                  | Collagen type IV (µg/µL) | Laminin (ng/mL) |
|----------------------------|-------------------------|-----------------|
| LPS (1µg/ml)               | 30.53±0.9               | 57.12±2.00      |
| LPS (10µg/ml)              | 29.17±0.36              | 56.61±2.39      |
| KCCM1 (unstimulated)       | 34.41±1.22              | 62.00±2.23      |
| KCCM2 (1µg/ml LPS-activated) | 42.39±1.74             | 69.40±1.86      |
| KCCM3 (10µg/ml LPS-activated) | 31.13±0.64            | 56.34±2.17      |
| KCCM1+anti-TGF-β1 antibodies | 32.18±1.71            | 58.87±2.31      |
| KCCM2+anti-TGF-β1 antibodies | 36.26±0.91            | 65.25±1.95      |
| KCCM3+anti-TGF-β1 antibodies | 30.76±1.88            | 56.16±2.62      |

**Figure 1** Effect of KCCM or LPS on ECM secretion by HSC.

**Stimulatory factor assay**
The concentration of TGF-β1 in LPS-activated KCCM was markedly higher (0.941±0.114) than that in unstimulated KCCM (0.465±0.075).

**Table 3** TGF-β1 in LPS-activated and unstimulated KCCM (mean±SD, n=6)

| Group                        | TGF-β1 (OD) |
|------------------------------|-------------|
| KCCM1 (unstimulated)         | 0.465±0.075 |
| KCCM2 (1µg/ml LPS-activated) | 0.941±0.114 |
| KCCM3 (10µg/ml LPS-activated)| 0.669±0.093 |
| Control group                | 0.089±0.010 |

**P<0.01 vs control group.**
the presence at a moderate concentration of LPS can release products, which stimulate HSC proliferation and collagen accumulation. These in vitro observations presumably mimic some profibrogenic situations in vivo, whereas HSC are activated in the presence of LPS.

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Edited by Zhang JZ and Wang XL