PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORTS •

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

Xin Zhang, Wei-Ping Yu, Lei Gao, Kai-Bin Wei, Jiu-Long Ju, Jia-Zhang Xu

Xin Zhang, Wei-Ping Yu, Kai-Bin Wei, Basic Medical School, Southeast University, Nanjing 210009, Jiangsu Province, China
Xin Zhang, Lei Gao, Jiu-Long Ju, Jia-Zhang Xu, 81 Hospital of PLA, Nanjing 210002, Jiangsu Province, China
Correspondence to: Dr. Wei-Ping Yu, Department of Pathology and Pathophysiology, Basic Medical School, Southeast University, Nanjing 210009, Jiangsu Province, China
Telephone: +86-25-3272508
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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-β_1 production in the KCCM was detected by enzyme-linked immunosorbent assay (ELISA).

RESULTS: HSC and Kupffer cells isolated had high purity. One microgram per mililiter 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 mililiter LPS-activated KCCM (0.106±0.010) was unable to promote HSC proliferation ($P>0.05$). Adding anti-TGF-β_1 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 mililiter LPS-activated KCCM contained a larger amount of TGF-β_1 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.

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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[7-9] 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[16-18], 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 0111: 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[19-25] 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 Ca^{2+}/Mg^{2+}-free Hank' s balanced salt solution (pH 7.4, 37℃) at a flow rate of 15 mL/min, and was then perfused with Hank' s balanced salt solution (pH 7.4, 37℃) 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℃ for 20 min. After passing through a filter (mesh size 74 μm), cells were washed twice with Hank' s balanced salt

solution and HSC were obtained by centrifugation over 12% (wt/vol) Nycodenz 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 1×10^5 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℃ in a humidified atmosphere containing 5% CO_2. The media of Kupffer cells were replaced 6 h after seeding and at every 48 h thereafter. Impurities caused by some endothelial cells were removed during the first medium change. The media of HSC were replaced 24 h after seeding and at every 48 h thereafter. Primary culture of HSC were allowed to grow to confluence 14 d after seeding, subcultured by trypsinization (0.125% trypsin) and then cultured in 96-well culture plates at a concentration of 1×10^5 cells/ml. HSC and Kupffer cells were identified by immunocytochemistry[26]. HSC were also identified by vitamin A typical fluorescence at 328 nm excitation wavelength.

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℃ 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-β_1 immunoglobulin. Conditioned media were collected and stored at -70℃ until use. The remaining cells were added with 180 μl media and 20 μl MTT (5 g/L) at 37℃. 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 optic-metric density (OD) was read on an ELISA reader at test wavelength of 570 nm and referent wavelength of 630 nm.

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-β_1 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 Nycodenz 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 refraction 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 proliferated 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-β_1 antibodies ($P<0.01$), but there was a difference compared with control group ($P<0.01$) (Table 1).

Table 1 Effect of KCCM or LPS on HSC proliferation (mean±SD, $n=12$)

Treatment	A
LPS (1 μg / ml)	0.096±0.003
LPS (10 μg / ml)	0.101±0.004
KCCM1 (unstimulated)	0.123±0.008[c]
KCCM2 (1 μg / ml LPS-activated)	0.132±0.005[a]
KCCM3 (10 μg / ml LPS-activated)	0.106±0.010[d]
KCCM1 + anti-TGF-β_1 antibodies	0.109±0.009[b]
KCCM2 + anti-TGF-β_1 antibodies	0.115±0.008[d]
KCCM3 + anti-TGF-β_1 antibodies	0.106±0.010
Control group	0.100±0.003

[a]$P<0.01$ *vs* control group; [b]$P<0.01$ *vs* unstimulated KCCM group; [c]$P<0.05$, [d]$P<0.01$ *vs* 1 μg / ml LPS-activated KCCM group.

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 30.53±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-β_1 antibodies. Ten microgram per mililiter 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).

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/L)	Laminin (ng/ml)
LPS (1 μg/ml)	30.53±0.9	57.12±2.00
LPS (10 μg/ml)	29.17±1.36	56.61±2.39
KCCM1 (unstimulated)	34.41±1.22[c]	62.00±2.23[c]
KCCM2 (1 μg/ml LPS-activated)	42.39±1.74[a]	69.40±1.86[a]
KCCM3 (10 μg/ml LPS-activated)	31.13±0.84[d]	56.34±2.17[d]
KCCM1+anti-TGF-β_1 antibodies	32.18±1.71[b]	58.87±2.31[b]
KCCM2+anti-TGF-β_1 antibodies	36.26±0.91[d]	65.25±1.95[d]
KCCM3+anti-TGF-β_1 antibodies	30.76±1.88	56.16±2.62
Control group	30.34±1.02	57.56±1.56

[a]P<0.01 *vs* control group; [b]P<0.01 *vs* unstimulated KCCM group; [c]P<0.05, [d]P<0.01 *vs* 1 μg/ml LPS-activated KCCM group.

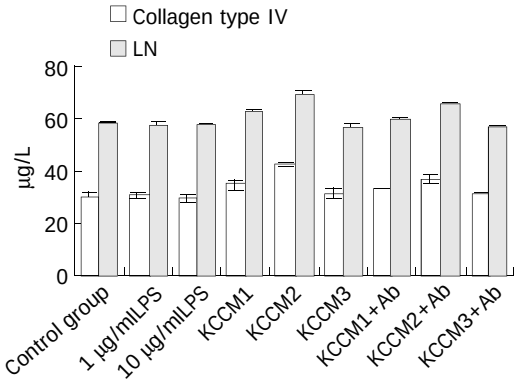

Figure 1 Effect of KCCM or LPS on ECM secretion by HSC.

Stimulatory factor assay

The concentration of TGF-β_1 in LPS-activated KCCM was markedly higher (0.941±0.114) than that in unstimulated KCCM (0.465±0.075).

Table 3 TGF-β_1 in LPS-activated and unstimulated KCCM (mean±SD, n=6)

Group	TGF-β_1(OD)
KCCM1(unstimulated)	0.465±0.075[a]
KCCM2(1 μg/ml LPS-activated)	0.941±0.114[a]
KCCM3(10 μg/ml LPS-activated)	0.669±0.093[a]
Control group	0.089±0.010

[a]P<0.01 *vs* control group.

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 mimiced 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-β_1 is the most powerful cytokine to promote fibrosis. During the period of HSC activation, TGF-β_1 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-β_1 antibodies, suggesting that HSC proliferation might be related with TGF-β_1 and some other cytokines, because anti-TGF-β_1 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-β_1, 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-β_1 than 1 μg/ml LPS, and secret other cytokines to counteract the effect of TGF-β_1.

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

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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