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Induction of hepatic stellate cell proliferation by LPS-stimulated peripheral blood mononuclear cells from patients with liver cirrhosis

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Abstract: We studied hepatic stellate cell proliferation in vitro. Peripheral blood mononuclear cells (PBMC) from patients with chronic active hepatitis C (CAH) and liver cirrhosis (LC) were cultured for 24 h in the presence or absence of *Escherichia coli* lipopolysaccharides (LPS). Hepatic stellate cell proliferation induced by the culture supernatants was measured, and interleukin-1 (IL-1) and IL-6 levels in the culture supernatants were quantified. Culture supernatants of LPS-stimulated PBMC from LC patients induced rat hepatic stellate cell proliferation by almost 2.8-fold (stimulation index, 2.83 ± 1.41) compared with when the cells were cultured without addition of PBMC culture supernatants. Production of IL-1β was significantly higher in the culture supernatants of both CAH and LC patients than in those of ten healthy controls ($P < 0.01$ and $P < 0.05$, respectively). But there was no significant correlation between IL-1 production and the induction of hepatic stellate cell proliferation by the culture supernatants. Although there were no significant differences in IL-6 production by LPS-stimulated PBMC among healthy controls and CAH and LC patients, we observed a significant correlation between IL-6 production and the induction of hepatic stellate cell proliferation in the culture supernatants of LC patients. Rat hepatic stellate cells themselves produced IL-6, and treatment with IL-6 antisense oligodeoxynucleotides suppressed the cell proliferation, suggesting that IL-6 is an autocrine growth factor for hepatic stellate cells. The addition of human recombinant IL-6 (hrIL-6) augmented rat hepatic stellate cell proliferation, indicating that excessive IL-6 may further facilitate cell proliferation. These findings suggest that a cytokine cascade including IL-6 may participate in hepatic stellate cell proliferation in LC patients when they are exposed to endotoxin.

Key words: liver cirrhosis, culture supernatant, hepatic stellate cell, interleukin-6

Introduction

Liver fibrosis is a complex process that involves the deposition of extracellular matrix components, the activation of cells capable of producing inflammatory mediators such as cytokines, and tissue remodelling. Excessive production of cytokines and inflammatory mediators has been reported in chronic liver diseases.[1–8] We previously found that the culture supernatants from lipopolysasccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) from liver cirrhosis (LC) patients induced significant fibroblasts proliferation. In this study, we demonstrated the mitogenic effect of culture supernatants from LC patients on isolated rat hepatic stellate cells (Ito cells); we suggest a possible role for interleukin-6 (IL-6) in this process.

Patients and methods

Patients

Fourteen patients with chronic active hepatitis (CAH), 16 LC patients who were infected with hepatitis C virus (HCV), and 10 age- and sex-matched healthy volunteers (controls) were studied.

The mean ages of the CAH and LC patients were 61 ± 8 and 61 ± 9 years, respectively. Informed consent was obtained from all participating healthy volunteers and patients before blood collection.

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PBMC isolation and culture

PBMC from heparinized venous blood were isolated by Ficoll-gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway), washed three times in Hanks' balanced salt solution (HBSS; GIBCO BRL, Life Technologies, Grand Island, NY, USA) and resuspended in RPMI 1640 medium (GIBCO) supplemented with 2 mM L-glutamine, 100 U/ml penicillin G (Pn), 100 μg/ml streptomycin (SM), and 10% fetal bovine serum (FBS; GIBCO) inactivated for 30 min at 56°C.

PBMC were adjusted to a concentration of 1×10^6/ml in 10-ml polypropylene tubes, and 1-ml aliquots of cell suspension were placed in a 24-well cell culture plate (Costar, Cambridge, MA, USA). PBMC were cultured for 24 h in 95% humidified air containing 5% CO_2 (37°C), in the presence or the absence of 10 μg/ml *Escherichia coli* lipopolysaccharides (LPS) (Sigma Chemical, St. Louis, MO, USA), and the supernatants were collected through a 0.45-μm millipore filter (Millex-HA Millipore, Tokyo, Japan) and stored at −20°C.

Isolation of hepatic stellate cells

Hepatic stellate cells (Ito cells) were isolated from the livers of male Wistar rats (350–450 g) as previously described, with minor modifications.[9–11] Liver was perfused in situ at 37°C at a flow rate of 10 ml/min, first with 125 ml of Libovitz L-15 medium (Sigma) containing 10 mmol/l HEPES, 1 U/ml penicillin G, and 1 μg/ml streptomycin, followed by perfusion with 100 ml of Eagle's minimum essential medium (EMEM) containing 200 mg of non-specific protease (Calbiochem—Novabiochem Corporation, La-Jolla, CA, USA), and the liver was then perfused extensively with 225 ml of EMEM containing 80 mg of collagenase (Serva Feinbiochemica, Heidelberg, Germany). After perfusion, the liver was excised, opened with scissors, and further digested in 100 ml of EMEM containing 20 mg protease and 1 mg DNase I (DTI; GIBCO) and agitated in a 37°C rotary shaking bath for 20 min at 275 rpm. The resulting cell suspensions were filtered through 60-μm nylon mesh. After being washed (1200 rpm, 5 min), the cells were resuspended in 45 ml EMEM containing 0.8 mg DNase I and layered across six tubes containing 3 ml of 28.7% Nycodenz (Sigma). After centrifugation at 3000 rpm for 20 min at 25°C, hepatic stellate cells were recovered at the interface between medium and Nycodenz, washed in 10 ml HBSS at 1200 rpm for 5 min, and suspended in EMEM containing 10% FBS. The concentration was adjusted to 1×10^6/ml, and cells were plated in six-well plates (Corning, Cambridge, MA, USA). The medium was replaced 24 h after plating, and every 24 h thereafter. Cells adhered and spread relatively slowly in primary culture. After 3 days of incubation, spreading was complete. After 7 days, the culture appeared as a dense homogeneous population of hepatic stellate cells. Desmin was used as a confirmatory marker of hepatic stellate cell purity.[12,13] Greater than 90% of the cells in primary hepatic stellate cell cultures displayed desmin immunofluorescence when stained with antiserum to this protein.

Proliferation of hepatic stellate cells in the presence of culture supernatants

Hepatic stellate cells were plated in 96-well flat-bottomed plates (Costar) in EMEM supplemented with 10% FBS at a concentration of 1×10^3 cells/well in a final volume of 200 μl. After overnight attachment, the culture medium was removed and replaced with 200 μl of FBS-free EMEM containing the 20% culture supernatants of PBMC. The cells were incubated for a total of 48 h, and were pulsed with 0.2 μCi ^{3}H-thymidine (^{3}H-TdR) during the last 24 h. Then cells were treated with trypsin/ethylenediaminetetraacetic acid (EDTA; GIBCO) and harvested onto glass filters the radioactivities were measured in a liquid scintillation counter.[14] Assays were done in triplicate. Hepatic stellate cell proliferation was evaluated by [^{3}H]-thymidine ([^{3}H]-TdR) incorporation and calculated as the stimulation index in order to detect the effect of LPS-stimulated and non-stimulated PBMC culture supernatants, using [^{3}H]-TdR incorporation of the hepatic stellate cells without PBMC culture supernatants as controls. The stimulation index was calculated as A/C and B/C, where:

A = [^{3}H]-TdR incorporation of rat hepatic stellate cells by the addition of LPS-stimulated PBMC culture supernatants (cpm)
B = [^{3}H]-TdR incorporation of rat hepatic stellate cells by the addition of LPS-nonstimulated PBMC culture supernatants (cpm)
C = [^{3}H]-TdR incorporation of rat hepatic stellate cells without the addition of PBMC culture supernatants (cpm)

Quantification of IL-1β and IL-6 in culture supernatants

We measured IL-1β and IL-6 levels with a commercially available solid-phase enzyme-linked immunosorbent assay (Human IL-1β and IL-6 detection kit; Toray TFB Tokyo, Japan) in accordance with the protocol supplied by the manufacturer. The standards and samples were incubated with a murine monoclonal antibody coating a 96-well microtiter plate for 2 h at room temperature. The wells were washed with buffer solution, 20% Tween 20 was added, and then incubation was done with horseradish peroxidase-conjugated and biotiny-

lated monoclonal antibody against IL-1β and IL-6, respectively, for 1 h at room temperature. The plates were washed, and reaction solution (tetramethylbenzydine [TMB]-H_2O_2) was added. After 15- to 30-min incubation at room temperature, the reaction was stopped with 1.8 N H_2PO_4 and the microtiter plates were read at 450 nm with a correction wavelength of 630 nm in a microtest-platereader (EAR 340T; SLT Lab Instruments, Austria). IL-1β and IL-6 concentrations were determined by absorbance, using a standard curve obtained with known quantities of IL-1β and IL-6 in the same assay. All samples were run in duplicate.

Effect of hepatic stellate cell proliferation and production of IL-6 after treatment with hrIL-6, IL-6 antisense (AS) or sense oligodeoxynucleotides

The effects of human recombinant IL-6 (hrIL-6) (ICN), IL-6 AS, or sense oligodeoxynucleotides on the proliferation of rat hepatic stellate cells were examined by [^{3}H]-thymidine uptake assay. IL-6 antisense oligodeoxynucleotides (5′-CCT CTC CGG ACT TGT GAA-3′) corresponding to a region from he second exon of the *IL-6* gene were synthesized (Sawady com, Tokyo, Japan). Sense oligodeoxynucleotides (5′-TTC ACA AGT CCG GAG AGG-3′) was used as controls. Hepatic stellate cells were cultured for 6 h in FBS-free EMEM, then exposed to IL-6 AS or sense olygodeoxynucleotides (10 μM) for 48 h; the medium was then changed to fresh EMEM containing 5% FBS, and the cells were cultured for 72 h. In another set of experiments, hrIL-6 (10 ng/ml) was included in cell cultures containing IL-6 AS oligodeoxynucleotides. The percent ^{3}H-TdR uptake without treatment was set as 100%.

Statistical analysis

Values for cytokine production were expressed as means ± SEM, and the effects on proliferation of rat hepatic stellate cells (stimulation index values) were expressed as means ± SD. Statistical analysis was performed with the two-tailed unpaired Student's *t*-test. Correlation testing was performed using linear regression. Results were considered statistically significant at $P < 0.05$.

Results

Proliferation of hepatic stellate cells in the presence of culture supernatants

The culture supernatants of LPS-stimulated PBMC from LC patients induced hepatic stellate cell proliferation by almost 2.8-fold (stimulation index, 2.83 ± 1.41), compared with when the cells were cultured without addition of PBMC culture supernatants; this was significantly higher than the stimulation indices for CAH and healthy controls (1.32 ± 0.22, 1.78 ± 0.23, respectively, $P < 0.05$).

There were no significant differences in hepatic stellate cell proliferation among LC, CAH, and healthy controls when the cells were incubated with the culture supernatants of non-stimulated PBMC from these three groups (stimulation indices were 1.54 ± 0.52, 1.28 ± 0.23 and 1.45 ± 0.19, respectively) (Table 1).

Cytokine production in culture supernatants

Spontaneous IL-1β production in the culture supernatants of non-stimulated PBMC from patients with CAH or LC (127 ± 44 [$n = 8$], 74 ± 18 [$n = 12$]; mean ± SE pg/ml, respectively) was similar to that in the culture supernatants from healthy controls (73 ± 18 [$n = 10$] pg/ml). IL-1β production in the culture supernatants of LPS-stimulated PBMC from patients with CAH or LC was significantly higher than in the culture supernatants of healthy controls (13.3 ± 2.6 [$n = 8$], 8.7 ± 1.9 [$n = 12$] ng/ml vs 4.0 ± 0.5 ($n = 10$) ng/ml; $P < 0.01$, $P < 0.05$, respectively) (Fig. 1).

Spontaneous IL-6 production in the culture supernatants of PBMC from patients with CAH or LC (0.42 ± 0.13 [$n = 14$], 0.91 ± 0.26 [$n = 16$]; mean ± SE ng/ml,

Table 1. Induction of proliferation of hepatic stellate cells by culture supernatants of LPS-stimulated PBMC from patients with chronic HCV infection

		A/C (LPS-stimulated)		B/C (LPS-non-stimulated)		A/B (Effect of LPS)	
Healthy controls	($n = 10$)	1.78 ± 0.23	$P < 0.05$	1.45 ± 0.19	N.S.	1.23 ± 0.08	$P < 0.01$
Chronic active hepatitis	($n = 14$)	1.32 ± 0.22		1.28 ± 0.23		1.05 ± 0.16	
Liver cirrhosis	($n = 16$)	2.83 ± 1.41		1.54 ± 0.52		1.80 ± 0.72	

Data values are expressed as stimulation indices (mean ± SD) relative to control without PBMC culture supernatants performed in triplicate wells. A, [^{3}H]-thymidine ([^{3}H]-TdR) incorporation of rat hepatic stellate cells by addition of LPS-stimulated PBMC culture supernatants (cpm); B, [^{3}H]-TdR incorporation of rat hepatic stellate cells by addition of LPS-non-stimulated PBMC culture supernatants (cpm); C, [^{3}H]-TdR incorporation of rat hepatic stellate cells cultured without addition of PBMC culture supernatants (cpm)
LPS, *Escherichia coli* lipopolysaccharides; HCV, hepatitis C virus; PBMC, peripheral blood mononuclear cells; NS, not significant

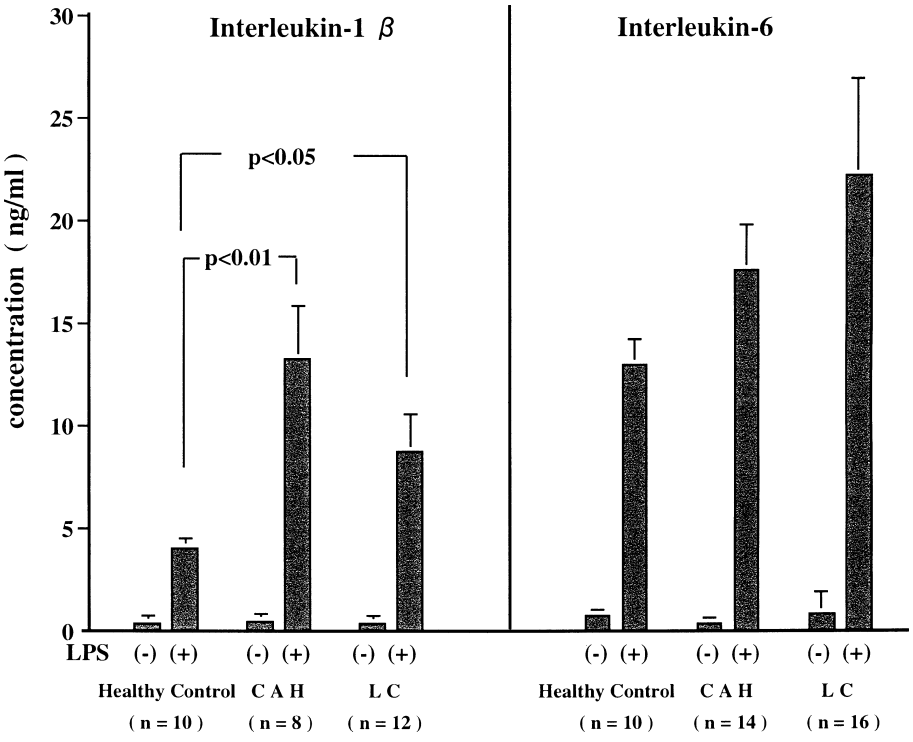

Fig. 1. Production of cytokines (interleukin [IL]-1β and IL-6) in culture supernatants of non-stimulated and lipopolysaccharide (*LPS*)-stimulated peripheral blood mononuclear cells (PBMC) from patients with hepatitis C virus (HCV)-infected chronic liver diseases (chronic active hepatitis [*CAH*] and liver cirrhosis [*LC*]). Culture supernatants were collected from non-stimulated and LPS-stimulated PBMC which were cultured for 24 h. IL-1β and IL-6 levels in culture supernatants were measured using solid-phase enzyme-linked immunosorbent assay (ELISA). Culture supernatants of LPS-stimulated PBMC from CAH and LC patients showed significantly increased IL-1β production compared with that in healthy controls ($P < 0.01$ and $P < 0.05$, respectively). Data values are expressed as means ± SE of triplicate experiments

respectively) was similar to that in the culture supernatants from healthy controls (0.81 ± 0.25 [$n = 10$] ng/ml). When PBMC were stimulated by LPS, IL-6 production in LC patients was somewhat higher than that in CAH patients and healthy controls (LC, 22.18 ± 4.65 [$n = 16$], CAH, 17.60 ± 2.19 [$n = 14$], healthy control, 12.98 ± 1.19 [$n = 10$] ng/ml, respectively), although there were no significant differences among them (Fig. 1).

Correlation between cytokine production and hepatic stellate cell proliferation

No correlation was observed between IL-1β production and hepatic stellate cell proliferation in the culture supernatants of LPS-stimulated PBMC from CAH and LC patients.

However, a significant correlation was observed between IL-6 production and hepatic stellate cell proliferation in the culture supernatants of LPS-stimulated PBMC from LC patients ($r = 0.720$; $P < 0.01$) (Fig. 2). No such correlation was observed for the culture supernatants of CAH patients and healthy controls.

Effects of hrIL-6 and IL-6 antisense oligodeoxynucleotides on hepatic stellate cell proliferation

Although hepatic stellate cells themselves produce IL-6 to some extent (Fig. 3), it has been unclear whether IL-6 is associated with the cell proliferation. We examined the effects of hrIL-6 and IL-6 AS oligodeoxynucleotides on the hepatic stellate cell proliferation. DNA synthesis by hepatic stellate cells was stimulated by the extrinsic addition of hrIL-6 to the culture medium (131.5 ± 3.3%; $P < 0.005$ vs no addition of hrIL-6) (Fig. 4). Interleukin-6 antisense oligodeoxynucleotides suppressed the IL-6 production of hepatic stellate cells (Fig. 3). The cell growth was also inhibited by the IL-6 AS oligodeoxynucleotides (52.4 ± 1.3%; $P < 0.005$ vs no IL-6 AS oligodeoxynucleotides) but not by the sense oligodeoxynucleotides (96.1 ± 4.5%), and the addition of hrIL-6 partially restored the inhibitory effect of IL-6 AS oligodeoxynucleotides (71.5 ± 8.3%; $P < 0.05$) (Fig. 4).

Discussion

Liver fibrosis is an important determinant of chronic liver disease because it leads to LC and an increased risk of hepatocellular carcinoma. Hepatic stellate cells function in the progression of liver fibrosis. Hepatic stellate cells are the main cell type expressing procollagen and laminin in human and experimental liver fibrogenesis.[15–17] During hepatic fibrogenesis, hepatic stellate cells proliferate and acquire a modified phenotype, which is considered to be transitional, myofibroblast-like, or activated.[18–20]

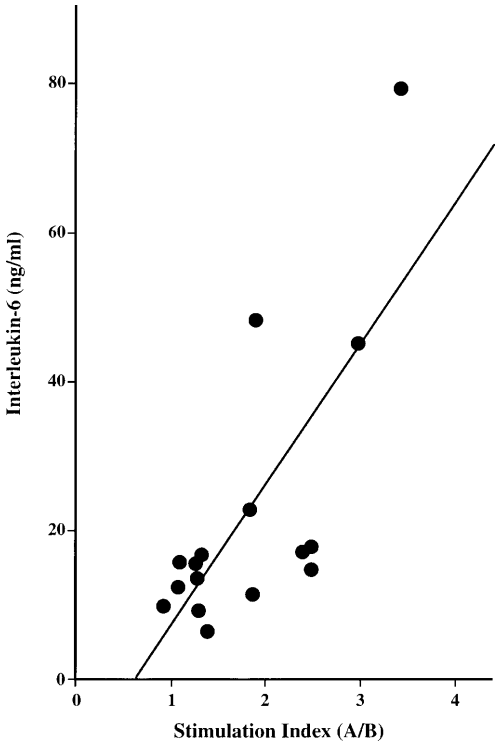

Fig. 2. Correlation between interleukin-6 production and rat hepatic stellate cell proliferation (stimulation index) by addition of culture supernatants of LPS-stimulated PBMC from LC patients; $n = 16$; $r = 0.720$; $P < 0.01$. See text for explanation of *stimulation index* (*A*/*B*)

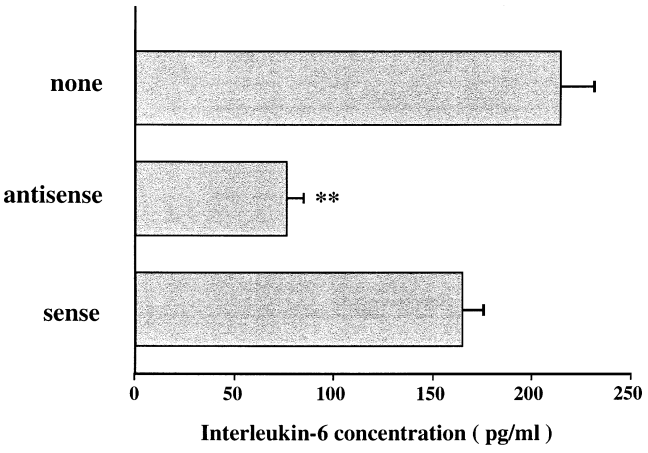

Fig. 3. IL-6 production by rat hepatic stellate cells. Treatment with IL-6 antisense oligodeoxynucleotides (*antisense*) suppressed the IL-6 production (**$P < 0.005$), whereas IL-6 sense oligodeoxynucleotides (*sense*) had no influence. *None*, No treatment with oligonucleotides

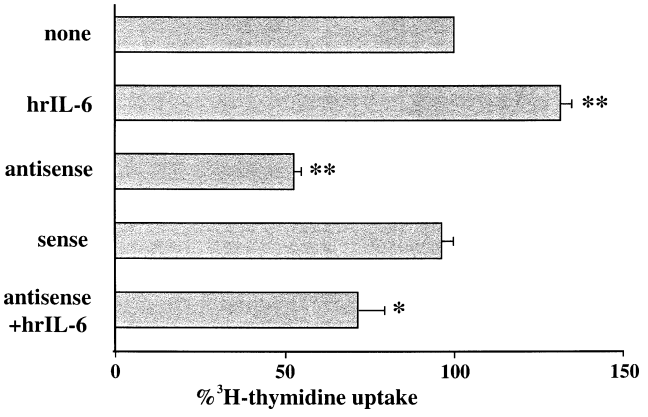

Fig. 4. Effect of human recombinant interleukin-6 (*hrIL-6*) and IL-6 antisense oligodeoxynucleotides (*antisense*) on proliferation of rat hepatic stellate cells. Data values are expressed as percent ^{3}H-thymidine uptake; the value without treatment (*none*) was set as 100%. Statistical analysis compared findings with and without treatment. **$P < 0.005$; *$P < 0.05$. *Sense*, IL-6 sense oligodeoxynucleotides

In this study, we demonstrated the mitogenic effect of LPS-stimulated PBMC cuture supernatant from LC patients on rat hepatic stellate cells. The culture supernatants from LC patients augmented the proliferation of rat hepatic stellate cells by almost 2.8-fold (2.83 ± 1.41) compared with when the cells were cultured without addition of PBMC culture supernatants. It has been reported that PBMC from patients with liver diseases show altered cytokine production, both spontaneously and in response to endotoxin, compared with controls.[21] We have also reported that the levels of IL-1β production in chronic HCV infection were significantly higher than those in healthy controls and that the IL-1β production was not correlated with the grade of hepatic fibrosis, suggesting the possibility that HCV infection of PBMC augments LPS-inducible cytokine production.[22] In this study, we found that some soluble factors secreted by PBMC after in vitro LPS stimulation induced rat hepatic stellate cell proliferation. IL-1β was excluded from the candidates because the IL-1β production in liver cirrhosis was equivalent to that in CAH in our experiments.[22] Transforming growth factor-β1 (TGF-β1) has been reported to have a mitogenic effect on the regulation of human hepatic stellate cell proliferation,[23,24] but TGF-β1 alone did not stimulate rat hepatic stellate cell proliferation.[25]

IL-6 is a pleiotropic cytokine produced by various types of cells, and acts on many kinds of target cells in immune, hematopoietic, and inflammatory systems. High levels of IL-6 were detected in the sera of patients with alcoholic LC,[26] hepatitis B virus infection,[27,28] and acute hepatitis.[29,30] It has been reported that collagen synthesis was increased in the liver tissues and in the culture supernatants of hepatic lipocytes isolated from rats treated with human recombinant IL-6.[31] We there-

fore measured the production of IL-6 in the culture supernatants of LPS-stimulated PBMC from LC patients, and examined the correlation with the induction of rat hepatic stellate cell proliferation. IL-6 synthesis was increased in patients with LC and the production of IL-6 was well correlated with the degree of rat hepatic stellate cell proliferation. Although hepatic stellate cells themselves produce some IL-6,[32] the addition of hrIL-6 to cultured hepatic stellate cells further augmented cell proliferation. The inhibitory effect of antisense IL-6 oligodeoxynucleotides on hepatic stellate cells also confirmed the role of IL-6 in the cell proliferation. These results suggest that IL-6 is an autocrine growth factor for rat hepatic stellate cells; the excessive IL-6 contained in the PBMC culture supernatant after LPS stimulation in patients with cirrhosis may further facilitate hepatic stellate cell proliferation, thereby enhancing the progression of liver fibrosis. However, hepatic stellate cell proliferation did not seem to be solely caused by IL-6, because in patients with chronic active hepatitis, there was no correlation between the production of IL-6 and the degree of proliferation of hepatic stellate cells. Taken together, our findings suggest that a cytokine cascade, including IL-6, after LPS stimulation may participate in hepatic stellate cell proliferation and liver fibrosis, and another proliferation cytokine may be present in the culture supernatant of LPS-stimulated PBMC from LC patients.

The in vitro activation with LPS may represent the in vivo endotoxemia that is observed during acute events such as sepsis. Patients with LC have a marked tendency to develop spontaneous bacteremia or bacterial infections such as peritonitis and pneumonia.[33,34] The PBMC (monocyte) overresponsiveness to endotoxin (LPS) observed in LC may be involved in the pathogenesis of the liver fibrosis. A better understanding of the mechanism of monocyte dysfunction is necessary to establish a specific treatment for liver fibrosis.

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