

In vitro expression of CD14 protein and its gene in Kupffer cells induced by lipopolysaccharide

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OBJECTIVES: To observe expression of CD14 protein and its gene in Kupffer cells induced by lipopolysaccharide (LPS) and explore the role of CD14 in LPS-induced Kupffer cell activation.

METHODS: Kupffer cells were isolated from Wistar rat livers by in situ collagenase digestion, followed by culture and incubation with 100 µg/ml LPS for 0, 30, 60 and 120 min, respectively. CD14 protein expressed on the membrane of Kupffer cells was examined using confocal microscopy and Western blotting analysis. Expression of CD14 mRNA in Kupffer cells was determined by reverse transcription polymerase chain reaction (RT-PCR). Tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the supernatant were measured using enzyme-linked immunosorbent assay (ELISA) kit.

RESULTS: Expression of CD14 mRNA and CD14 protein in isolated Kupffer cells was upregulated quickly after LPS stimulation and increased with time. Likewise, there was a time-dependent increase of TNF-α and IL-6 in the supernatant with upregulation of CD14 expression. There were significant differences between normal and LPS-stimulated Kupffer cells ($P < 0.05$).

CONCLUSIONS: LPS can upregulate expression of CD14 protein and its gene in isolated Kupffer cells. CD14 may play an important role in the activation of LPS-induced Kupffer cells.

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Key words: lipopolysaccharide; CD14; Kupffer cells

Introduction

CD14, a 55-kDa glycoprotein, was first described as a differentiation antigen on the surface of myeloid lineage cells.^[1] It acts as a glycosylphosphatidylinositol (GPI)-anchored receptor

for the complex of lipopolysaccharide (LPS) and LPS binding protein (LBP) and plays a key role in the activation of LPS-induced monocytes/macrophages.^[1,2] Kupffer cells as special macrophages residing within hepatic sinusoids are an important defensive barrier for gut-derived endotoxemia and are activated in the course of LPS clearance. It is reported that excessive release of cytokines from LPS-activated Kupffer cells contributes to organ damage during sepsis.^[3] So investigation of LPS-induced signal transduction in Kupffer cells may be helpful in exploring new therapeutic tool for sepsis and septic shock caused by gram-negative bacteria. However, whether CD14 acts, as it does in other cells, as an important signaling molecule for LPS-induced Kupffer cell activation is still unclear. The purpose of the present study was to observe the relation among LPS stimulation, Kupffer

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cell activation and CD14 expression in Kupffer cells, and further evaluate the role of CD14 in LPS-induced Kupffer cell activation.

Methods

Isolation of Kupffer cells

Adult Wistar rats each weighing between 200 g and 240 g were used in this study. Kupffer cells were isolated from the rat liver by *in situ* collagenase digestion, as described previously with slight modification.^[4] Briefly, the liver was excised after perfusion through the portal vein first with Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution (HBSS), then with HBSS containing 0.05% collagenase IV (Sigma, USA). The nonparenchymal cells fraction was separated from parenchymal cells by centrifugation at 50 g for 3 min, then was centrifuged on a 50:25% Percoll gradient at 1000 g for 15 min to obtain the Kupffer cell fraction. The viability of isolated cells measured by trypan blue exclusion was >95%, and the purity of Kupffer cells indicated by phagocytosis of latex beads was >90%.

Cell culture and treatment

Isolated Kupffer cells were cultured in 24-well plates at a concentration of 1×10^6 /well and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate) at 37 °C in 5% CO_2 for 24 h. Subsequently they were incubated with 100 $\mu\text{g}/\text{ml}$ LPS for 0, 30, 60 and 120 min, respectively. There were four samples at each time point. After incubation, the supernatant was collected and stored at -20 °C until use; Kupffer cells were digested using 0.25% trypsin and were used for the following detections.

RT-PCR analysis for CD14 mRNA

Total RNA was isolated from Kupffer cells with trizol reagent (Life Technologies, USA). All materials used for RNA isolation were treated with diethylpycarbonate (DEPC) (Sigma, USA) followed by vapour sterilization. Each total RNA sample was reverse-transcribed to complementary DNA (cDNA) using RT-PCR kit (Roche, USA). cDNA

was stored at -70 °C until PCR. Specific sense and anti-sense primers used for PCR were as follows: 5'-CTCAACCTAGAGCCGTTTCT-3' and 5'-CAGGATTGTCAGACAGGTCT-3' (CD14); 5'-ATGGGGAAGGTGAAGGTCGGA-3' and 5'-TTC-CACGATACCAAAGTTGTCA-3' (GAPDH, internal standard). The expected sizes of amplified PCR product were 267bp for CD14 and 576bp for GAPDH. The conditions for amplification were as follows: denaturation at 93 °C for 1 min, annealing at 57 °C for 1 min, and extension at 70 °C for 2 min for 30 cycles. The PCR product was then separated by 1.5% agarose gel electrophoresis and was visualized by staining the gel with ethidium bromide. The relative amount of amplified cDNA was quantified from relative optical density of the band, using a bio-image analysis system (Bio-Rad Gel Doc 2000, USA).

Laser scanning confocal microscopy

Kupffer cells were grown on glass coverslips, fixed in 4% paraformaldehyde solution, and rinsed by PBS for three times. The cells were incubated first with rabbit anti-rat CD14 polyclonal antibody (Santa Cruz, USA) at 37 °C for 60 min, then with FITC conjugated goat anti-rabbit IgG (Santa Cruz, USA) for 30 min. CD14 protein was visualized by confocal microscopy (Leica TCS NT 160195, Germany).

Western blotting analysis for CD14 protein

Membrane protein extracts of the cultured Kupffer cells were obtained by homogenizing samples in a cell lysis buffer containing 20 mmol HEPES (pH 7.9), 25% glycerol, 0.42 mmol NaCl, 15 mmol MgCl_2 , 0.2 mmol EDTA, 0.5 mmol phenylmethylsulfonyl fluoride (PMSF) and 0.5 mmol dithiothreitol (DTT), then by two cycles of centrifugation at 12 000 g for 15 min. Protein concentration was determined using a Bradford assay kit (Bio-Rad, Hercules, CA, USA). Extracted protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes (Dupont, USA). Blocked by PBS-Tween containing 5% skim milk, membranes were incubated first with

Table 1. Expression of CD14 mRNA in Kupffer cells induced by 100 µg/ml LPS at different times (ROD) (n=4)

Time (min)	0	30	60	120
CD14 mRNA	1.79 ± 0.34	8.32 ± 1.29 *	13.01 ± 2.15 *	24.67 ± 3.76 *

* : P < 0.05 vs 0 min.

Table 2. Expression of CD14 protein on Kupffer cells induced by 100 µg/ml LPS at different times (ROD) (n=4)

Time (min)	0	30	60	120
CD14	8.44 ± 1.26	33.69 ± 4.35 *	40.59 ± 6.03 *	45.44 ± 7.29 *

* : P < 0.05 vs 0 min.

Table 3. Levels of TNF-α and IL-6 in the supernatant of Kupffer cells induced by 100 µg/ml LPS at different times (pg/ml) (n=4)

Time (min)	0	30	60	120
TNF-α	55.75 ± 6.86	215.56 ± 28.32 *	488.54 ± 58.15 *	1286.50 ± 134.36 *
IL-6	163.15 ± 25.67	346.42 ± 40.75 *	665.36 ± 74.86 *	1424.33 ± 156.48 *

* : P < 0.05 vs 0 min.

rabbit anti-rat CD14 polyclonal antibody (Santa Cruz, USA), then with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, USA). Finally, the membranes were developed with diaminobenzidine reagent. The relative amount of CD14 protein was quantified from relative optical density of the band, using a bio-image analysis system (Bio-Rad Gel Doc 2000, USA).

Enzyme-linked immunosorbent assay

The supernatant was collected and stored at -20 °C until ELISA analysis. TNF-α and IL-6 in the supernatant were quantified using specific Enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Cambridge, MA, USA). Each sample was tested in duplicate.

Statistical analysis

Results were expressed as mean ± SD. Statistical difference was calculated by analysis of variance (ANOVA) using SAS software. A P value of less than 0.05 was considered significant.

Results

Expression of CD14 mRNA in Kupffer cells

Little expression of CD14 mRNA could be found in normal Kupffer cells (0-min group). However,

this expression was significantly upregulated in Kupffer cells at 30 min and increased with the time after LPS stimulation (Table 1).

Expression of CD14 protein on the membrane of Kupffer cells

Laser scanning confocal microscopy showed that there was little FITC-CD14 positive cells before LPS stimulation, but the number of FITC-CD14 positive cells and fluorescence intensity increased with time after LPS induction, indicating

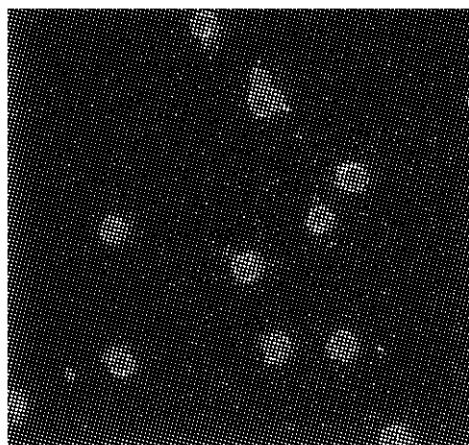


Fig. Expression of CD14 protein on Kupffer cells was significantly upregulated at 120 min after LPS stimulation.

increasing expression of CD14 protein on the membrane of Kupffer cells (Fig.). Likewise, Western blotting analysis showed that there was little expression of CD14 protein on the membrane of normal Kupffer cells, but the amount of CD14 protein increased significantly at 30 min and continued to increase after LPS stimulation (Table 2).

Levels of TNF- α and IL-6 in supernatant

The TNF- α and IL-6 were low in the supernatant of normal Kupffer cells. However, they were increased significantly at 30 min and continued to increase with time after Kupffer cells were induced by LPS (Table 3).

Discussion

It is well known that LPS is much responsible for the pathogenesis of sepsis and septic shock caused by gram-negative bacteria. LPS exerts its effects mainly by activating monocytes/macrophages, leading to synthesis and release of toxic mediators from activated cells.^[5,6] CD14 is a receptor for the LPS-LBP complex, expressed on the surface of many types of cells, such as monocytes, macrophages, hepatocytes and liver sinusoidal endothelial cells, and it plays a pivotal role in LPS-induced cell activation by functioning as a key signaling molecule.^[1,2,7,8] Kupffer cells are special macrophages residing in hepatic sinusoids, which constitute 80%–90% of total fixed macrophages in the body. Because of their special location, Kupffer cells act as an important defensive barrier for gut-derived endotoxemia by clearing LPS from portal circulation and are activated at the same time. Excessive release of toxic mediators from LPS-activated Kupffer cells will contribute to organ damage during early sepsis.^[3] Hence investigating of LPS-induced signal transduction in Kupffer cells may be helpful in exploring new therapeutic tool for sepsis and septic shock caused by gram-negative bacteria. But so far, whether LPS receptor CD14 functions as a key signaling molecule for LPS-induced Kupffer cell activation is still controversial. Ikejima et al^[9] reported that Kupffer cells from estrogen-treated animals expressed more CD14 than normal con-

trols, leading to greater sensitivity of Kupffer cells to LPS and more production of toxic mediators. In addition, Su et al^[10] found that CD14 mRNA was increased in Kupffer cells from animal models of alcoholic liver disease, and that increased expression of CD14 correlated with the presence of pathological liver injury. These results indicate that CD14 is a key signaling molecule for LPS-induced Kupffer cell activation, because Kupffer cells activated by gut-derived endotoxin play an important role in alcoholic liver damage.^[11] On the other hand, Bellezzo et al^[12] reported that NF-kappa B in Kupffer cells was activated by LPS under CD14-independent conditions, and the activation was not potentiated by serum containing LBP. Furthermore, it has been reported that CD14 expression was very low in Kupffer cell even after LPS stimulation, and LPS-induced TNF- α release by Kupffer cells was CD14 independent.^[13] These reports suggest that CD14 is an insignificant factor for LPS signaling pathway in Kupffer cells.

In view of the contradictory opinions, we observed the relation between LPS-induced Kupffer cell activation and expression of CD14 in these cells. We found that expression of CD14 protein and CD14 mRNA was very low in normal Kupffer cells but was upregulated by LPS in a time-dependent manner. As a mark of Kupffer cell activation, production of TNF- α and IL-6 was not obvious in normal Kupffer cells, but it was markedly enhanced with upregulation of CD14 in LPS-induced Kupffer cells. So our results also suggest that CD14 may be a key signaling molecule for LPS-induced Kupffer cell activation.

Why there were reports that Kupffer cells lack CD14-mediated LPS signaling pathway?^[12,13] We think there are two reasons. One is that CD14 is not an unique receptor for LPS. In addition to CD14, scavenger receptor and CD11/18 act as LPS receptors on the surface of monocytes/macrophages, and CD11/18 also can initiate LPS signal to cell, resulting in cell activation.^[2,14] The other is the method of Kupffer cell isolation. Routinely, Kupffer cells are isolated by *in situ* collagenase digestion, but some investigators use additional reagents such as pronase in order to increase Kupffer

cell yield. However, recent study^[15] has found that pronase can destroy CD14 on Kupffer cells in a short time. We consider that under normal conditions CD14 plays a predominant role in LPS-induced Kupffer cell activation in comparison with CD11/18. When CD14 is destroyed in the course of Kupffer cell isolation, CD11/18 may become a substitute for CD14, leading to the phenomenon that LPS-induced Kupffer cell activation is independent of CD14.

In the present study, we used collagenase alone during cell isolation and protected CD14 on Kupffer cells. According to our results and the report^[16] that CD14 knockout mice were less susceptible to alcohol-caused liver injury, we conclude that LPS can upregulate CD14 expression in Kupffer cells, and CD14 may be a key signaling molecule for LPS-induced Kupffer cell activation.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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