Expression of toll-like receptor 4 and MD-2 gene and protein in Kupffer cells after ischemia-reperfusion in rat liver graft

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Abstract

AIM: To investigate the expression of toll-like receptor 4 (TLR4) and MD-2 gene and protein in Kupffer cells (KCs) and their role in ischemia-reperfusion (IR) injury of rat liver graft.

METHODS: KCs were isolated at 0 (control group), 2, 12, 24 h (IR group) following IR in rat liver graft. mRNA expression of TLR4 and MD-2 was detected by RT-PCR analysis, protein expression of TLR4/MD-2 was detected by flow cytomteric (FCM) analysis, and tumor necrosis factor-α (TNF-α) level in supernatant was measured by ELISA. Then isolated KCs were incubated with anti-TLR4 polyclonal antibody (anti-TLR4 group), and TNF-α level was measured again.

RESULTS: The mRNA and protein expression of TLR4/MD-2 and the level of TNF-α in IR group increased significantly at 2 h following IR, and reached the maximum at 12 h, and slightly decreased at 24 h, but were still significantly higher than those in the control group (P<0.01). The expression of these factors was markedly decreased after anti-TLR4 antibody treatment as compared with the IR group (P<0.01).

CONCLUSION: Lipopolysaccharide (LPS) following IR can up-regulate TLR4/MD-2 gene and protein expression in KCs, and synthesize cytokine TNF-α. Anti TLR4 antibody can inhibit the production of TNF-α induced by LPS. TLR4 and its partner molecule MD-2 may play an important role in Kupffer cell activation and IR injury.

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INTRODUCTION

Toll-like receptor 4 (TLR4) is a transmembrane protein, mainly existed in monocytes/macrophages[1-3]. It plays an important role in recognizing endotoxin (lipopolysaccharide, LPS) or LPS-CD14 complex, and mediating monocyte/macrophage activation and pro-inflammatory cytokine release[2,4]. MD-2 molecule is a secreting protein, linking with the exocytic area of TLR4, and facilitating the binding of TLR4 and LPS to enhance cell activation induced by LPS[6-10]. Kupffer cells (KCs) are not only the biggest residing macrophages in the body, but also are the main participant of ischemia-reperfusion injury (IRI) in liver transplantation[11-14]. Therefore, studying the expression of TLR4 and MD-2 gene and protein in KCs following IR is very important to clarify the source of cytokines involved in IRI. The aim of the current study was to investigate the expression of TLR4 and MD-2 gene and protein, and the production of cytokines in liver graft by isolating and culturing KCs following IRI, and to probe the role of TLR4 and MD-2 in IRI injury.

MATERIALS AND METHODS

Establishment of animal model of IRI following liver transplant in rat

Male Wistar rats weighing 210-250 g, supplied by Chongqing Medical University, were used as donors and recipients. Orthotopic liver transplantation was performed with improved two-cuff technique introduced by Peng et al.[15] as following: (1) Graft procurement: portal vein (PV), hepatic artery (HA), infrahepatic vena cava (IHVC), and bile duct were divided. Then the graft was infused and harvested for preparation. PV and IHVC were prepared by cuff technique, and a drainage Teflon catheter was inserted into the bile duct. (2) Implantation of graft: the graft was placed orthotopically in the recipient’s abdominal cavity after the original liver was removed. The suprahepatic vena cava was sutured end to end, and the portal vein was anastomosed by cuff method, then the liver was perfused. After the anastomosis of IHVA and HA was completed, the bile duct was reestablished end to end by Teflon catheter.

KC isolation and culture

KCs were isolated at 0, 2, 12, 24 h following IR with in situ collagenase perfusion technique described by Gong et al.[16]. In brief, the liver was removed with portal vein perfusion with Hank’s balance salt solution (HBSS) and digested in a solution of 0.5 g/L collagenase (type IV, Sigma, USA). The digest was washed thoroughly and plated on plastic dishes in RPMI medium containing 100 mL/L fetal calf serum (FCS). After 6 h incubation at 37°C in humidified atmosphere containing 50 mL/L CO2 in air, the nonadherent cells were removed by pipet. The adherent cells were collected with a rubber policeman. KC purity exceeded 90% as assessed by light microscopy, and viability was greater than 95% as determined by trypan blue exclusion assay. The KCs at 0 h were regarded as control group, and those at 2, 12 and 24 h were as IR group.

RNA isolation and complementary DNA synthesis

Total RNA was isolated from rat KCs using the TRIZOL reagent (Life Technologies, USA). The quality of RNA was controlled by the intactness of ribosomal RNA bands. A total of 5 µg of each intact total RNA sample was reverse-transcribed to complementary DNA (cDNA) using AMV reverse transcriptase (Roche, USA).

Determination of TLR4 and MD-2 mRNA by RT-PCR

The PCR primers used are shown in Table 1. The conditions for
amplification were as follows: denaturation at 94 °C for 1 min,
annealing at 58 °C for 1 min, and extension at 72 °C for 1 min for
30 cycles, and extension at 72 °C for 7 min again. The PCR products
were electrophoresed on 20 g/L agarose gels containing ethidium bromide, and video photographed on an ultraviolet
transilluminator, and the results were shown as the ratio of relative absorbance of TLR4 or MD-2/β-actin analyzed with
Quantity one 4.1.0 software.

Table 1 Primer used for PCR amplification of TLR4 and MD-2
gene

| Primer sequence       | Size (bp) |
|-----------------------|-----------|
| TLR4                  | 311       |
| 5′ TGGATACGTTCTTATAAG 3′ |          |
| 5′ GAAATGGAGGACACCCCTT3′ |          |
| MD-2                  | 332       |
| 5′ ATGGTCTTCTGGCGAGTTT3′ |          |
| 5′ CCATGCGCAAGACCTCCTT3′ |          |
| β-actin               | 300       |
| 5′ ATCATGTGGAGACCTCACA 3′ |          |
| 5′ CACTTCTGCTGAAGTCCA 3′ |          |

Flow cytometric analysis
Expression of TLR4 and MD-2 membrane protein in KCs was
determined by flow cytometric analysis. In brief, KCs were
incubated with rabbit anti-rat TLR4 polyclonal antibody (1 µg/mL)
labeled with FITC and goat anti-rat MD-2 polyclonal antibody
(1 µg/mL) labeled with PE (Sigma, USA). After washed three
times, 10 000 cells were analyzed by flow cytometry (Coulter,
USA), and the percentage and mean fluorescence intensity
(MFI) of TLR4 and MD-2-positive cells were taken as the indexes.

TNF-α level in supernatant
Supernatants were collected to measure the levels of TNF-α
with ELISA kit (Jingmei Biotechnology Company, China)
following the manufacturer’s instructions.

Blocking test of anti-TLR4 antibody
To determine the role of TLR4 and MD-2 in LPS-induced KC
activation, KCs were harvested and adjusted to the concentration
of 1×10⁶/mL. Then, 0.2 mL of anti-TLR4 antibody (1:100)
was added to the medium, and incubated for 30 min at 37 °C.
The supernatant was collected again for measuring the levels
of TNF-α by ELISA analysis following the manufacturer’s instructions.

Statistical analysis
All results were expressed as mean±SD. Statistical difference
between means was determined by Student’s t test using SigmaPlot 2000 software (SPSS Inc., USA). A P value <0.01 was
considered statistically significant.

RESULTS
Expression of TLR4 and MD-2 mRNA in KCs
RT-PCR analysis showed that KCs from control group had low
but detectable levels of TLR4 and MD-2 mRNA. The mRNA
level was significantly increased with time, reaching its maximum
of nine-fold at 12 h following IR, and slightly declined 24 h after
IR, but was still significantly higher than that in the control
group (P<0.01) (Figure 1).

Binding of FITC, PE to KCs
To confirm the expression of TLR4 and MD-2 membrane protein
on KCs, we examined the binding of FITC/PE to the cells. TLR4
and MD-2 positive cells were 7.94% and 6.51% in control group.
But in the rats with IR, the mean fluorescence intensity (MFI)
was significantly increased, and TLR4 positive cells were 45.71%,
78.46%, and 61.72% and PE positive cells were 38.64%, 74.15%,
and 58.47%, respectively, at 2 h, 12 h and 24 h following IR,
which were significantly higher compared with the control group
(P<0.01) (Figure 2).
Decay of TNF-α levels after anti-TLR4 treatment

In IR group, the level of TNF-α in supernatant of KCs was 267.4±24.1, 529.1±30.9, and 329.7±76.4 ng/mL at 2, 12 and 24 h after IR, respectively. In group of anti-TLR4 antibody blockade, the production of TNF-α in supernatant was obviously inhibited by Ab against TLR4 when compared with that in IR group (P<0.01), and it was 102.5±10.4, 207.6±18.4, and 185.3±12.7 ng/mL at 2, 12 and 24 h after IR, respectively (Figure 3).

DISCUSSION

Toll protein is a transmembrane protein which mainly acts as a necessary tool for immune defence to microorganisms[6,17-20]. The first toll-like protein in human cells was reported by Janeway, and named toll-like receptor (TLR)[19,20]. About ten members of TLR superfamily in mammals have been reported in recent years. Among them, TLR4 plays a key role in LPS signal transduction[15-22,23].

When LPS enters blood, it first binds to LBP (LPS binding protein), forming a LPS-LBP compound, and then binds to TLR4 on cell membranes leading to activation of cells[18,24,25]. Recent studies have confirmed that the activation of cells induced by LPS needs another important molecule, namely MD-2. MD-2, and subsequently release harmful cytokines, which can induce activation of KCs by combining with TLR4 and MD-2, and subsequently release harmful cytokines, which can induce activation of KCs by combining with TLR4 and MD-2. When LPS enters blood, it first binds to LBP (LPS binding protein), forming a LPS-LBP compound, and then binds to TLR4 on cell membranes leading to activation of cells[18,24,25]. Recent studies have confirmed that the activation of cells induced by LPS needs another important molecule, namely MD-2. MD-2, and subsequently release harmful cytokines, which can induce activation of KCs by combining with TLR4 and MD-2. Kupffer cells are special macrophages residing in hepatic sinusoids, which constitute 80% to 90% of total fixed macrophages in the body[21,22,23,24]. Because of their special location, KCs act as an important defensive barrier for gut-derived endotoxemia. A large amount of endotoxins contact with KCs through portal circulation following ischemia-reperfusion after liver transplantation, and activate the KCs[25,31]. Thus, it is very important to study the expression of TLR4 and MD-2 in KCs to clarify the origin of cytokines and the mechanism of IRI.

In our study, we found there were weak expressions of TLR4 and MD-2 gene and protein in resting KCs, which might play a critical role in maintaining the normal function of KCs, and might be essential for keeping KCs to be ready for activation after stress. But after IR, a large mount of LPS entered liver sinusoids, activated KCs, resulting in increased release of harmful cytokines such as TNF-α, which reached the maximum at 12 h after IR. To correctly illuminate the relation between TLR4 and MD-2 and downstream cytokines, we used anti-TLR4 antibody to inhibit the binding function of TLR4, and found that the production of TNF-α was greatly decreased, suggesting that the expression of TLR4 and MD-2 protein is indispensable to the secretion of downstream cytokines.

In summary, the present data suggest that there are weak expressions of TLR4 and MD-2 mRNA and protein in Kupffer cells. Expressions of TLR4 and MD-2 can significantly increase following ischemia-reperfusion in rat liver transplantation. LPS can induce activation of KCs by combining with TLR4 and MD-2, and subsequently release harmful cytokines, which ultimately attack the graft. Further studies are needed for elucidating TLR4/MD-2-mediated signal transduction pathways which lead to the secretion of cytokines during IRI on liver graft.

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REFERENCES

1 Latz E, Visintin A, Lien E, Monks B, Golenbock DT. The LPS receptor generates inflammatory signals from the cell surface. J Endotoxin Res 2003; 9: 375-380
2 Espevik T, Latz E, Lien E, Monks B, Golenbock DT. Cell distributions and functions of Toll-like receptor 4 studied by fluorescent gene constructs. Scand J Infect Dis 2003; 35: 660-664
3 Kimoto M, Nagasawa K, Miyake K. Role of TLR4/MD-2 and RI015/MD-1 in innate recognition of lipopolysaccharide. Scand J Infect Dis 2003; 35: 569-572
4 Harada K, Ohiro S, Ise K, Ozaki S, Zen Y, Sato Y, Nakanuma Y. Lipopolysaccharide activates nuclear factor-kappaB through toll-like receptors and related molecules in cultured biliary epithelial cells. Lab Invest 2003; 83: 1657-1667
5 Fujihara M, Muroi M, Tanamoto K, Suzuki T, Azuma H, Ikeda H. Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. Pharmacol Ther 2003; 100: 171-194
6 Re F, Strominger JL. Separate functional domains of human MD-2 mediate Toll-like receptor 4 binding and lipopolysaccharide responsiveness. J Immunol 2003; 171: 5272-5276
7 Guillot L, Medjane S, Le-Barilie R, Balley V, Daniel C, Chignard M, St-Tahar M. Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. J Biol Chem 2004; 279: 2712-2716
8 Asai Y, Hashimoto M, Ogawa T. Tepponemal glycoconjugates inhibit Toll-like receptor ligand-induced cell activation by blocking LPS-binding protein and CD14 functions. Eur J Immunol 2003; 33: 3196-3204
9 Totemeoyer S, Foster N, Kaiser P, Maskell DJ, Bryant CE. Toll-like receptor expression in CH/HeN and CH/HeJ mice during Salmonella enterica serovar Typhimurium infection. Infect Immun 2003; 71: 6653-6657
10 Akashi S, Saitoh S, Wakabayashi Y, Kikuchi T, Takamura N, Nagai Y, Kusumoto Y, Fukase K, Kusumoto S, Adachi Y, Kosugi A, Miyake K. Lipopolysaccharide interaction with cell surface Toll-like receptor 4:MD-2: higher affinity than that with MD-2 or CD14. J Exp Med 2003; 198: 1035-1042
11 Schauer RJ, Gerbes AL, Vonier D, Meissner H, Michl P, Leiderer R, Schildberg FW, Messmer K, Bilzer M. Glutathione protects the rat liver against reperfusion injury after prolonged warm ischemia. Ann Surg 2004; 239: 220-231
12 Cavalleri B, Perrelli MG, Aragno M, Ramadori P, Poli G, Cutrin JC. Ischaemic preconditioning modulates the activity of Kupffer cells during in vivo reperfusion injury of rat liver. Cell Biochem Funct 2003; 21: 299-305
13 Carini R, Albano E. Recent insights on the mechanisms of liver preconditioning. Gastroenterology 2003; 125: 1480-1491
14 Giakoustidis DE, Stavrou A, Antoniou I, Kontos N, Kostopoulou E, Botsoglou NA, Gerasimidis T, Iliadis S, Tsantilas D, Papageorgiou G, Argyropoulos CJ. Ischaemic preconditioning modulates the activity of Kupffer cells during in vivo reperfusion injury of rat liver. Cell Biochem Funct 2003; 21: 299-305
15 Carini R, Albano E. Recent insights on the mechanisms of liver preconditioning. Gastroenterology 2003; 125: 1480-1491
16 Giakoustidis DE, Stavrou A, Antoniou I, Kontos N, Kostopoulou E, Botsoglou NA, Gerasimidis T, Iliadis S, Tsantilas D, Papageorgiou G, Argyropoulos CJ. Ischaemic preconditioning modulates the activity of Kupffer cells during in vivo reperfusion injury of rat liver. Cell Biochem Funct 2003; 21: 299-305
17 Carini R, Albano E. Recent insights on the mechanisms of liver preconditioning. Gastroenterology 2003; 125: 1480-1491
18 Giakoustidis DE, Stavrou A, Antoniou I, Kontos N, Kostopoulou E, Botsoglou NA, Gerasimidis T, Iliadis S, Tsantilas D, Papageorgiou G, Argyropoulos CJ. Ischaemic preconditioning modulates the activity of Kupffer cells during in vivo reperfusion injury of rat liver. Cell Biochem Funct 2003; 21: 299-305
19 Uehori J, Matsumoto M, Tsuji S, Akazawa T, Takeuchi O, Akira S, Kawata T, Azuma I, Toyoshima K, Seya T. Simultaneous blocking of human Toll-like receptors 2 and 4 suppresses myeloid dendritic cell activation induced by Mycobacterium bovis bacillus Calmette-Guerin peptidoglycan. Infect Immun 2003; 71: 4238-4244
20 Vives-Pi M, Somoza N, Fernandez-Alvarez J, Vargas F, Caro P, Alba A, Gomis R, Labeta MO, Pujol-Borrell R. Evidence of expression of endotoxin receptors CD14, toll-like receptors TLR4 and TLR2 and associated molecule MD-2 and of sensitivity to ...
endotoxin (LPS) in islet beta cells. *Clin Exp Immunol* 2003; 133: 208-218

21 Tamai R, Sugawara S, Takeuchi O, Akira S, Takada H. Synergistic effects of lipopolysaccharide and interferon-gamma in inducing interleukin-8 production in human monocytic THP-1 cells is accompanied by up-regulation of CD14, Toll-like receptor 4, MD-2 and MyD88 expression. *J Endotoxin Res* 2003; 9: 145-153

22 Suzuki M, Hisamatsu T, Podolsky DK. Gamma interferon augments the intracellular pathway for lipopolysaccharide (LPS) recognition in human intestinal epithelial cells through coordinated up-regulation of LPS uptake and expression of the intracellular Toll-like receptor 4-MD-2 complex. *Infect Immun* 2003; 71: 3503-3511

23 Thompson PA, Tobias PS, Viriyakosol S, Kirkland TN, Kitchens RL. Lipopolysaccharide (LPS)-binding protein inhibits responses to cell-bound LPS. *J Biol Chem* 2003; 278: 28367-28371

24 Ohnishi T, Muroi M, Tanamoto K. MD-2 is necessary for the toll-like receptor 4 protein to undergo glycosylation essential for its translocation to the cell surface. *Clin Diag Lab Immunol* 2003; 10: 405-410

25 Miyake K, Nogai Y, Akashi S, Nagafuku M, Ogata M, Kosugi A. Essential role of MD-2 in B-cell responses to lipopolysaccharide and Toll-like receptor 4 distribution. *J Endotoxin Res* 2002; 8: 449-452

26 Medvedev AE, Vogel SN. Overexpression of CD14, TLR4, and MD-2 in HEK 293T cells does not prevent induction of *in vitro* endotoxin tolerance. *J Endotoxin Res* 2003; 9: 60-64

27 Okamoto M, Sato M. Toll-like receptor signaling in anti-cancer immunity. *J Med Invest* 2003; 50: 9-24

28 Schroder NW, Morath S, Alexander C, Hamann L, Hartung T, Zahringer U, Gobel UB, Weber JR, Schumann RR. Lipoteichoic acid (LTA) of Streptococcus pneumoniae and Staphylococcus aureus activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J Biol Chem* 2003; 278: 15587-15594

29 Zhu X, Qiu Y, Shi M, Ding Y. Matrine protects sinusoidal endothelial cells from cold ischemia and reperfusion injury in rat orthotopic liver transplantation. *Ann Clin Lab Sci* 2003; 33: 216-225

30 Kojima Y, Suzuki S, Tsuchiya Y, Konno H, Baba S, Nakamura S. Regulation of pro-inflammatory and anti-inflammatory cytokine responses by Kupffer cells in endotoxin-enhanced reperfusion injury after total hepatic ischemia. *Transpl Int* 2003; 16: 231-240

31 Kiemer AK, Gerwig T, Gerbes AL, Meissner H, Bilzer M, Vollmar AM. Kupffer-cell specific induction of heme oxygenase 1 (hsp32) by the atrial natriuretic peptide-role of cGMP. *J Hepatol* 2003; 38: 490-498

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