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Original Article

Expression of cartilage glycoprotein 39 in peripheral blood monocytes of septic rat and its role in TLR 4-NF-κB signaling pathways

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Abstract: Objective: To investigate Cartilage glycoprotein 39 (Cgp-39) expression in peripheral blood monocytes of septic rats, and analyze the relationship between Toll-like receptor 4 (TLR4)-NF-κB signalling pathway and Cgp-39 expression. Methods: The ligation puncture was performed to establish rat sepsis model, and ELISA was used to measure serum Cgp-39 concentration. Peripheral blood mononuclear cells was isolated and cultured for 72 h. RNA interference technology was used to inhibit TLR4 and NF-κB gene expression, and real-time PCR and Western blot were performed to detect mRNA and protein expression of TLR4 and NF-κB. Results: At 1 h, there was no significant differences in serum Cgp-39 concentration between sepsis group and the control group ($P > 0.05$), however, at 6 h, 12 h, 24 h and 48 h, serum Cgp-39 concentrations in sepsis group were significantly higher than those in the control group at the corresponding time points ($P < 0.05$). Compared with the control group, TLR4 mRNA and protein expression were increased significantly in sepsis group and sepsis NF-κB interference group; NF-κB mRNA and protein expression were increased significantly in sepsis group and sepsis TLR4 interference group. However, compared with sepsis group, Cgp-39 concentrations decreased significantly in either sepsis TLR4 interference group or NF-κB interference group ($P < 0.05$ for both). Conclusion: Cgp-39 is highly expressed in peripheral blood monocytes of septic rat and TLR4-NF-κB signalling pathways may be involved in the regulation of Cgp-39 expression.

Keywords: Sepsis, peripheral blood monocytes, cartilage glycoprotein 39 (Cgp-39), inflammatory factor, toll-like receptor 4 (TLR4), NF-κB

Introduction

Sepsis is a major cause of death in critically ill patients. Until now, there are no suitable biomarkers, which are sensitive and specific for diagnosis and can be used to guide the treatment of sepsis. Cartilage glycoprotein 39 (Cgp-39), known as a new kind of inflammatory markers [1], has been found involved in acute and chronic inflammatory process, but its relationship with sepsis and the related mechanism is not known yet. In this study, we tested Cgp-39 expression in peripheral blood monocytes of septic rats, and analyzed the relationship between Toll-like receptor 4 (TLR4)-NF-κB signalling pathway and Cgp-39 expression. This will provided insight of the relationship between Cgp-39 and sepsis.

Materials and methods

Reagents

SD rats were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China); Saline solution, 3% sodium pentobarbital and Hank's fluid were purchased from Sangon Biological Engineering Co. LTD (Shanghai, China); anticoagulant tube were purchased from Hehua Biological Engineering co., LTD (Shanghai, China); centrifuge tube (50 mL) were purchased from Corning

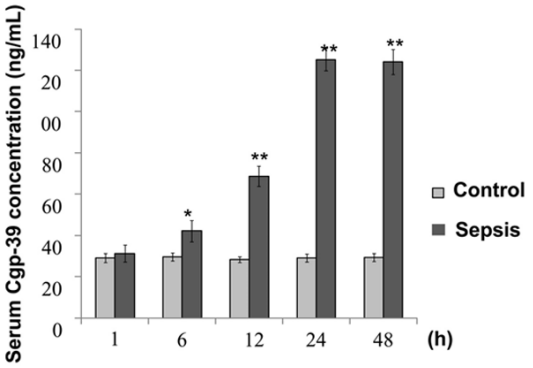

Figure 1. The serum concentrations of Cgp-39 (ng/mL) in sepsis group and the control group at different time point. (*P < 0.05, **P < 0.01).

Incorporated (Beijing, China); LTS1077 mononuclear cell separation fluid bought from Sigma (Beijing, China).

Sepsis model establishment and grouping

40 healthy SD rats (20 male and 20 female), were randomly divided into control group (10 rats) and sepsis group (30 rats). Sepsis rats were induced by cecal ligation and puncture (CLP). The protocols are as follows: the animals were housed with fasting and free drinking water 12 h before operation. Firstly, rats were intraperitoneal injected with 0.5 mL of 3% pentobarbital sodium. After aseptic laparotomy, cecum was isolated, ligated at the junction of the small intestine and large intestine and punctured twice at the end of the cecum with 9 size needle. Then abdominal cavity was closed. After operation, 5 mL/100 g dose of saline was subcutaneous injected to supply body fluid loss in the process of operation. The peripheral blood was drawn at 1 h, 6 h, 12 h, 24 h, and 48 h after operation. The plasma level of LPS, TNF alpha, IL-6 and whole blood level of white blood counts (WBC) were tested according to the reference [2]. The data from successful model rats entered the final data analysis. The standard of successful sepsis model is as followings: (1) The form of infection presented as localized infection, wound infection or endogenous infection; (2) the blood bacteria culture or bacterial endotoxin test is positive in the process of disease; (3) there are certain period of time from primary attack to multiple organ dysfunction syndrome (MODS); (4) there are typical sepsis manifestation, presented as high mobile cycling state and continuous high metabolic state; (5) there is enough mortality and incidence of MODS.

Separation of peripheral blood monocytes

Anticoagulant whole blood (5 mL) was transfered into a 50 mL pointed- bottom tube and centrifuged at 2000 RPM for 20 min. After removing the supernatant, about 2.5 mL lower precipitation cell was left. According to the volume ratio of 1:1, 2.5 mL Hank's liquid was added to suspend the precipitation. Then the suspended cells were slowly transferred into a 15 ml pointed-bottom centrifuge tube with 5 ml of LTS1077 lymphocyte separation medium inside using capillary suction pipe and make sure the diluted blood located above the lymphocyte separation liquid. After centrifuged at 2000 r/min for 20 min, the top layer of the plasma was removed. The monocytes between plasma layer and lymphocyte separation medium were collected. The monocytes suspension were then diluted consistently with 3-4 times volume of Hank's liquid using capillary suction pipe, avoiding the forming of tiny bubbles and ensuring that liquid column height is not more than two-thirds of the centrifuge tube. After blended, the suspension was centrifuged at 1500 r/min for 10 min. This procedure was repeated 2 more times. The cells were washed with RPMI-1640 Medium.

Construction of TLR4 and NF-κB specific siRNA recombined Lentivirus vector

TLR4 and NF-κB specific siRNA restructuring Lentivirus vector were constructed according to the method of literature [3] and named as TLR4 siRNA and NF-κB siRNA respectively.

Enzyme-linked immunosorbent assay

1 mL of blood collection taken from different time (1, 6, 12, 24 and 48 h) were transferred into 1.5 mL EP tube and incubated under the condition of 37°C for 30 min, and then the serum were transferred to another 1.5 mL EP tube and centrifuged at 3000 g for 10 min. The supernatant were collected and tested using the Cgp-39 ELISA Kit (item no: 8020, bought from Quidel Company) for each rat's serum samples. Cgp-39 concentration was also tested for both CLP group and the control group.

Cell culture and experiment designing

1 ml blood samples from the separation of monocytes were suspended with Hank's medium containing 20% calf serum and incubated

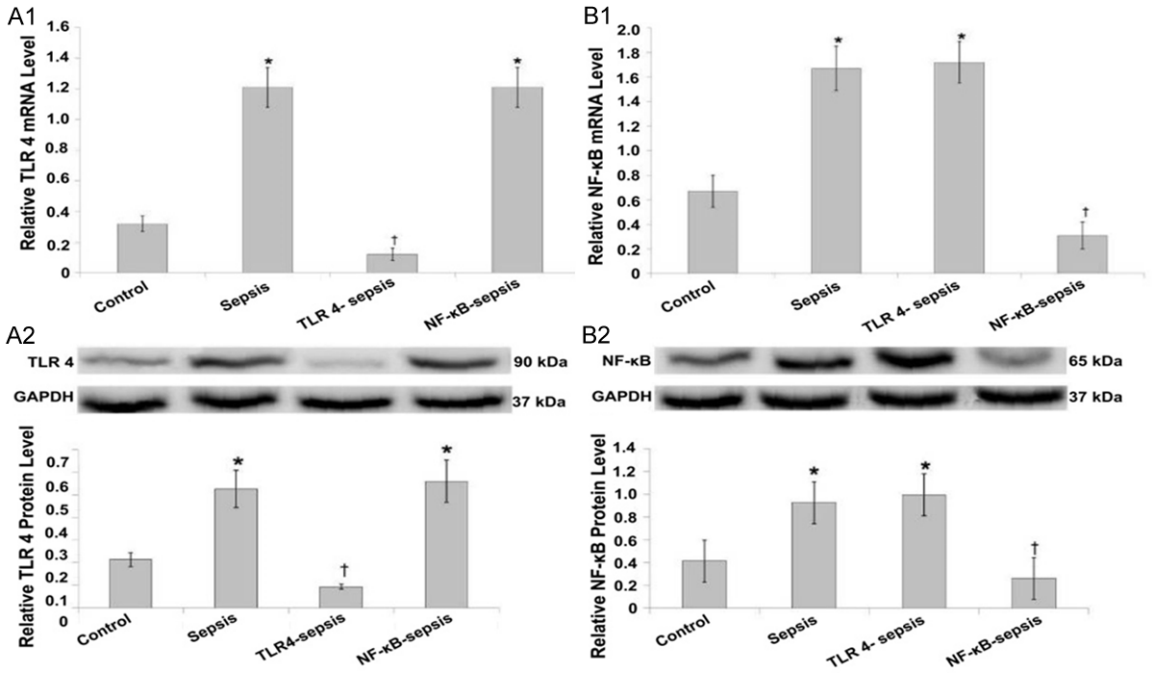

Figure 2. TLR4 and NF-κB expression in rats peripheral blood monocytes. A1. Fluorescence quantitative PCR detection of TLR4 mRNA amount; A2. Western blot detection of TLR4 protein expression; B1. Fluorescence quantitative PCR detection of NF-κB mRNA amount; B2. Western blot detection of NF-κB protein expression. *Compared with control group, $P < 0.05$; †Compared with sepsis group, $P < 0.05$.

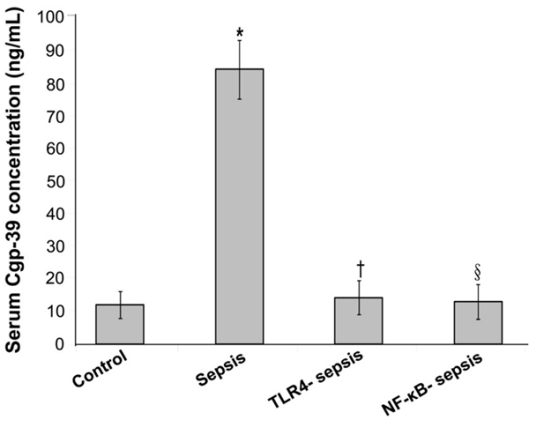

Figure 3. Cgp-39 expression in peripheral blood monocytes of each group. *Compared with control group, t = 17.83, $P = 0.000$; †Compared with sepsis group, t = 16.49, $P = 0.000$; §compared with sepsis group, t = 16.75, $P = 0.000$).

for 2-3 h at 37°C with 5% CO_2 in incubator. The adherent monocytes were collected after removing the supernatant. 3-4 mL RPMI-1640 medium containing 10% fetal bovine serum was added into each flask. Groupings are processed as the followings:

Control group: normal control group rats PBMC cell, without any processing, incubated for 72 h at 37°C and 5% CO_2; Sepsis groups: sepsis group PBMC, without any processing, incubated for 72 h at 37°C and 5% CO_2; Sepsis TLR4 interfering group: sepsis group PBMC, transfected with TLR4 siRNA (5×10^4 TU/MI), incubated for 72 h; Sepsis NF-κB interfering groups: sepsis group PBMC, transfected with NF-κB siRNA 5×10^4 TU/mL, incubated for 7 h. Samples were triplicated when tested using enzyme-linked immune methods .

Real time PCR

Rat peripheral blood nomocytes were isolated at different time (1, 6, 12, 24 and 48 h) and the total mRNA were extracted using TRIzol Reagent kit and diluted to 5 μg/μL with DEPC water. The total mRNA was reverse transcribed into cDNA using PrimeScript ® One Step RT-PCR Kit.

The specific primers is: GAPDH F: 5'-TGGTGAAGGTCGGTGTGAAC-3', GAPDH R: 5'-GCTCCTGGAAGATGGTGATGG-3'; TLR-2 F: 5'-CGCTTCCTGAACTTGTCC-3'; TLR-2 R: 5'-GGTTGTCACCTGCTTCCA-3'; The NF-κB F: 5'-CTGAACCAGGGCATACCTGT-3', the NF-κB R: 5'-GAGAAGTCCATGTCCGCAAT-3' (Sangon Co., Ltd, Shanghai). GAPDH were used as internal control, and SYBR Green fluorescence quantitative PCR kit

and IQ5 PCR amplification system (Bole Co. Ltd., Shanghai) was used to amply the tissue factor gene cDNA fragments and GAPDH gene fragment. The reaction condition were set as followings: 95°C degenerating for 5 min, 94°C degenerating 30 s, 57°C annealing 1 min, 72°C extending 30 s, 30 cycle, 72°C extending for 10 min. TF and GAPDH delta Ct were calculated for each sample and TF mRNA transcription factor for each samples were calculated using the delta Ct method [4].

Western blot

Rat peripheral blood monocytes from different time (1, 6, 12, 24 and 48 h) were suspended with buffer solution to 5×10^{10}/mL. Supernatant were collected after centrifuged at 12000 RPM for 20 min and prepared for electrophoresis. 5% and 15% separating gel was used to separate protein and electrically transferred protein to PVDF membrane. PVDF membrane were blocked with 10% milk for 1 h, incubated with first antibody at 37°C for 1 h, then washed with PBST 3 times (5 min for each time), then incubated with diluted second antibody solution at 37°C for 1 h, and washed with PBST three times (5 min for each time). Finally using ECL + plus™ Western blotting system kit (Amersham, USA) and 3490 Photo Gel Imaging Systems (Epson, Japan) developed and recorded the image. The relative amounts of each protein and GAPDH were analyzed with Image Pro PLUS software. The first antibody (1:1000 diluted): Rabbit anti-mouse TLR4 (No: EPNCIR133, Epitomics company, USA), Rabbit anti-rat NF-κB (No: ab31481, Abcam company, USA), Rabbit anti-rat GAPDH (item no: ab9484, Abcam, USA); Secondary antibody (1:5000 diluted): Goat anti-rabbit IgG (sc-2004, Santa Cruz, USA).

Statistical analysis

SPSS19.0 software was used for statistical analysis. The t test was used for all continuous data. Results were presented with the average ± standard error ($\bar{x}$ ± SEM). Differences were considered significant when $P < 0.05$.

Results

Cgp-39 expression in serum of septic rats

Serum Cgp-39 concentration at different time in sepsis group and the control group were shown in **Figure 1**. In sepsis group, the Cgp-39 concentration increases gradually with time, while in control group, there is no significant change in serum concentration of Cgp-39. There was no significant difference ($P > 0.05$) in the serum concentration of Cgp-39 at 1 h between sepsis group and the control group, while the serum concentration of Cgp-39 in sepsis group were significantly higher ($P < 0.05$) than in control group at 6 h, 12 h, 24 h and 48 h.

TLR4 and NF-κB expression in rats peripheral blood monocytes

TLR4 and NF-κB expression level and protein level were tested after 72 h of transfection in both TLR4-interfered sepsis group and NF-κB-interfered sepsis group. The results are shown in **Figure 2**. Compared with control group, TLR4 mRNA and protein level were significantly higher ($P < 0.05$) in sepsis group sepsis and NF-κB-interfered sepsis group, while TLR4 mRNA and protein level are significantly lower ($P < 0.05$) in TLR4-interfered sepsis group. Moreover, Compared with control group, NF-κB mRNA and protein level were significantly higher ($P < 0.05$) in sepsis group sepsis and TLR4-interfered sepsis group, while NF-κB mRNA and protein level were significantly lower ($P < 0.05$) in NF-κB-interfered sepsis group.

Cgp-39 expression in rats peripheral blood monocytes in rats peripheral blood monocytes

Cgp-39 concentration after 72 h of transfection in both TLR4-interfered sepsis group and NF-κB-interfered sepsis group are shown in **Figure 3**. In sepsis group, Cgp-39 concentration was significantly higher than that of control group ($P < 0.05$), while in NF-κB -interfered sepsis mRNA and TLR4-interfered sepsis group, Cgp-39 concentration was significantly lower than that of sepsis group ($P < 0.05$).

Discussion

Cgp-39 is a type of carbohydrate-binding protein and attributed to chitinase family. Its molecular weight is 40 kDa [5] and its crystal structure has been illuminated, but its biological function is still not very clear. Recent researches showed that Cgp-39 does not exist in normal human peripheral blood monocytes and its level rises only after infection, which indicated that it can be used as a kind of acute

phase inflammatory proteins [6]. Our previous studies showed that Cgp-39 were highly expressed in the blood samples from sepsis patients and decreased to about 40 percent after 12 h of continuous blood purification (CBP) treatment and decreased to negative after 24 h of CBP (normal value is 102 μg/L). In addition, the secretion of Cgp-39 is closely associated with some inflammatory cytokines. One study shows that IL-6 is a key factor for the Cgp-39 secretion in sepsis [7].

TLRs/LRRR signaling pathway is widespread in cell signal transduction pathways and plays important functions in the occurrence of many diseases such as inflammation and tumor development [8]. The molecular biology characteristic TLRs/NF-κB signal pathways are mainly presented in toll-like receptors family and the NF-κB protein family. TLRs are congenital immunological pathogenic pattern recognition receptors and play a key role in body defence against biological invasion. It constructed a bridge between the natural immune defence and acquired immune system. After the TLRs binding to pathogen, recognition molecules eventually lead to the activation of the NF-κB. NF-κB is a fast reactive transcription factors and commonly existed in the cytoplasm, which located downstream of the TLRs signaling pathways and involved in immune responses, cell proliferation and differentiation process [9, 10].

Sepsis is due to severe infections, such as bacterial infection from severe burns, multiple traumas, surgery. Lipopolysaccharide of bacterial and infection induced heat shock protein (HSP) released from necrotic cells can activate the TLR4 and combine with them. NF-κB, as important transcription factors in the cell, controls the activation of many molecules, which participate in every stage of the early immune response and inflammatory reaction, such as the secretion and expression of TNF-α, IL-β, IL-2 [11]. Many studies have shown that widespread LPS from Gram negative bacteria can activate the downstream signaling molecules such as MyD88 and IRAK through TLR4 pathway [12]. The cytokines expression regulated by TLR4-NF-κB will decrease if the expression of TLR4 or NF-κB is inhibited.

This study explored the correlation between the TLRs/NF-κB signaling pathway and Cgp-39 expression in rat models. We separated the rat peripheral blood monocytes at different time points and detected TLR4 and NF-κB mRNA and protein expression level using fluorescence quantitative PCR and Western blot. We found that increased Cgp-39 concentration in septic rat peripheral blood monocytes along with the increased TLR and NF-κB mRNA and protein level. Moreover, inhibiting TLR4 and NF-κB mRNA and protein level using siRNA, resulted in the Cgp-39 expression suppressed significantly in sepsis group. This study showed that the peripheral blood Cgp-39 is an acute phase reactant in sepsis, and its levels can be used to predict the occurrence of sepsis. Suppression of TLR4- NF-κB pathway induced the decreased expression of Cgp-39, which indicated TLR4-NF-κB pathway are activated in rats peripheral blood monocytes and regulated the expression of Cgp-39.

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Disclosure of conflict of interest

None.

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