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(Citation)

The Kobe journal of the medical sciences, 53(5):219-227

(Issue Date)

2007-01

(Resource Type)

departmental bulletin paper

(Version)

Version of Record

(JaLCDOI)

<https://doi.org/10.24546/81000121>

(URL)

<https://hdl.handle.net/20.500.14094/81000121>



Decreased Expression of Toll-like Receptor 2 and 4 on Macrophages in Experimental Severe Acute Pancreatitis

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Received 24 January 2007 /Accepted 5 February 2007

Key words: severe acute pancreatitis, TLR2, TLR4, expression, macrophage

In severe acute pancreatitis (SAP), immunologic impairment in the early phase may be linked to subsequent infectious complications that are main contributor to the high mortality. Toll-like receptors (TLRs) recognize microorganisms as the innate immune system, and are involved in host defense mechanism. TLR2 recognizes lipoteichoic acid (LTA) of gram-positive bacteria, and TLR4 recognizes lipopolysaccharide (LPS) of gram-negative bacilli. This study aimed to investigate the expression of TLRs on macrophages and their TLRs-mediated cytokine production in rat SAP. SAP was induced by retrograde injection of 3% sodium deoxycholate into the biliopancreatic duct in male Wistar rats. Macrophages were isolated from bronchoalveolar lavage fluid 6 hours after induction of SAP. The expression of TLR2 and TLR4 was analyzed by real-time RT-PCR and western blotting. TNF- α release from macrophages was estimated after 4-hour stimulation of LTA or LPS. Endotoxin/bacterial translocation (E/BT) was also evaluated in this model. The expression of TLR2 (mRNA and protein) and LTA-mediated TNF- α production were significantly decreased in SAP compared with control. The expression of TLR4 (mRNA and protein) and LPS-mediated TNF- α production was also significantly decreased in SAP compared with control. E/BT occurred 18 hours after induction of SAP. These results suggest that the impaired responsiveness to LTA and LPS of macrophages is derived from decreased expression of TLR2 and TLR4, respectively. This suppression of immune response in the early phase may be implicated in the mechanism of infectious complications.

Infectious complication (sepsis due to infected pancreatic necrosis) is the most serious complication in the late phase of severe acute pancreatitis (SAP), and contributes to the high mortality in this disease (2,4). This complication is thought to be a result of the endotoxin/bacterial translocation (E/BT) from the gastrointestinal tract (20,12). As an important mechanism of infection, we have reported that immunologic impairment (systemic immunosuppression) in the early phase is linked to the increased susceptibility to subsequent infection and the development of septic complications (25).

The innate immune system recognizes pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents, but not on the host. Toll-like receptors (TLRs), transmembrane proteins of the interleukin (IL)-1 receptor superfamily, recognize PAMPs and mediate the production of cytokines necessary for the development of effective immunity (13,24). Activation of TLRs leads not only to the induction of inflammatory

responses but also to the development of antigen-specific adaptive immunity. The TLR family consists of at least 10 members. TLR2 recognizes peptidoglycan and lipoteichoic acid (LTA) of gram-positive bacteria and lipoproteins of both gram-negative and gram-positive bacteria (1, 30). TLR4 recognizes lipopolysaccharide (LPS) of gram-negative bacilli (8, 13,18).

Recently, evidence has accumulated of the significance of TLRs in various pathological conditions such as sepsis (27), inflammatory bowel disease (7,16), surgical stress (9), and obstructive jaundice (15). Since the innate immune system operates mainly via TLRs on macrophages, it is conceivable that expression of TLRs on macrophages and their responsiveness to the agonists are of great importance for inflammatory response and host defense mechanism in SAP. However, they have not yet been examined. This study aimed to investigate the expression of TLRs (TLR2 and TLR4) on macrophages and their capacity of TLRs-mediated cytokine production in rat SAP.

MATERIALS AND METHODS

Animals. Male Wistar rats (weighing 300-350 g, 9 weeks old) were purchased from Charles River Japan Inc. (Yokohama, Japan). The protocol for this animal experiment was approved by the Institutional Animal Committee of Kobe University Graduate School of Medicine.

Model for acute necrotizing pancreatitis. Under anesthesia with diethyl ether, a midline laparotomy was performed, and the biliopancreatic duct was ligated at the orifice to the duodenum. Then, the biliopancreatic duct was cannulated with polyethylene tubing (PE-10, Becton Dickinson Co., Sparks, MD), and 100 μ l of 3% (wt/vol) sodium deoxycholate was injected retrogradely under low pressure with the temporary clamp of common bile duct at the hilus of the liver. Sham operation was performed in control rats. Ten ml of saline was injected subcutaneously immediately after the operation.

Experimental design. Rats were sacrificed 6 hours after induction of SAP and macrophages were isolated from bronchoalveolar lavage fluid (BALF). Expression of TLR2 and TLR4 (mRNA and protein) and agonist-induced (LTA for TLR2 and LPS for TLR4) cytokine production were analyzed. Blood sample and mesenteric lymph nodes (MLNs) were obtained 0, 6, 12, and 18 hours after induction of SAP.

Preparation of macrophages from BALF. A cannula was inserted into the trachea and secured with a suture. Lungs and trachea were lavaged with 1 ml of 37°C PBS. The lavage was repeated 10 times with the same PBS by instilling and withdrawing slowly. The BALF was centrifuged at 400 x *g* for 10 min at 4°C, and the pellet was resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS). The cells were cultured on plate for 2 hours, and washed with cold PBS to remove non-adhesive cells. Then, EDTA was added to the plate on ice and cells were collected. The recovery rate of macrophages was over 95%. After washing with PBS, macrophages were resuspended in RPMI 1640 containing 10% FBS.

Real-time RT-PCR. GenElute™ Mammalian Total RNA kit (Sigma Corp., Saint Louis, MO) was applied to isolate RNA from macrophages as described on the manufacture's protocol. SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA) was applied to synthesize cDNA from RNA as described on the manufacture's protocol. cDNAs were amplified by using the quantitative real-time PCR (ABI Prism 7000 Sequence Detection System; Applied Biosystems, Foster City, CA) with TaqMan Universal PCR Master Mix Reagent (Applied Biosystems, Foster City, CA). The specific primers and detection probes for TLR2 and TLR4 were synthesized from published GenBank sequences (Nippon EGT CO., Toyama, Japan). β -Actin TaqMan™ Endogenous

Control Kit (EUROGENTEC, Seraing, Belgium) was used as housekeeping gene. PCR conditions for all primers were as follows: 95°C for 15 seconds, and 60°C for 45 seconds for 40 cycles. Relative expression levels in the samples were calculated from TLR2 and TLR4 levels normalized to the β -Actin level. The primers were used as follows: TLR2 upstream, CAT CGA AAA GAG CCA CAA AAC TG; TLR2 downstream, GGT AGG TCT TGG TGT TCA TTA TCT TG; TLR2 probe, AAA GCC ATT CCC CAG CGC TTC TGC; TLR4 upstream, GCA TCA TCT TCA TTG TCC TTG AGA; TLR4 downstream, CTC CCA CTC GAG GTA GGT GTT T; TLR4 probe, AGG CAG CAG GTC GAA TTG TAT CGC C.

Western blotting. Macrophages were resuspended in lysate buffer (100 mM Hepes pH 7.5, 10% sucrose, 0.1% CHAPS, 1 mM EDTA, 10 mM DTT, 1 mM APMSF, 10 μ g/ml pepstatin, and 10 μ g/ml leupeptin) on ice for 30 minutes, and sonicated for 30 seconds three times. The lysate was centrifuged and the supernatant was collected. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories Inc., Hercules, CA). The lysates were treated with SDS-PAGE loading buffer (65 mM Tris, 5% 2-mercaptoethanol, 3% SDS, and 10% glycerol) at 95°C for 5 minutes. Fifty μ g of protein was electrophoresed on e-PAGEL (E-R7.5L; ATTO, Tokyo, Japan), and were transferred to PVDF membrane (Millipore Co., Bedford, MA). After blocking for 1 hour at room temperature, membranes were incubated with anti-human TLR2 rabbit polyclonal antibody or anti-mouse TLR4 goat polyclonal antibody (Santa Cruz Bio. Inc., Santa Cruz, CA) at a dilution of 1:100 overnight at room temperature. After washing, membranes were incubated with secondary HRP-conjugated goat anti-rabbit IgG or donkey anti-goat IgG at a dilution of 1:10000 for 30 minutes. After washing, the protein was detected by chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Pierce Bio. Inc., Rockford, IL) and Lumino Imaging Analyzer FAS-1000 (Toyobo Co., Osaka, Japan).

Measurement of TNF- α concentration. Macrophages (10³ cells/well) were stimulated with LTA (0–10³ ng/ml) or LPS (0–10⁵ ng/ml) (Sigma Corp., Saint Louis, MO). The culture medium was collected 4 hours after the incubation. The concentrations of TNF- α were determined with a rat TNF- α ELISA Kit (BioSource International, Inc. Camarillo, CA) according to the manufacturer's protocol.

Blood endotoxin level. Blood sample was quantitated for endotoxin using standard clinical automated analyzer.

Bacterial culture. MLNs were obtained under sterile conditions, and were processed for culture of aerobic and anaerobic organisms. Specimens were inoculated onto agar plates including BTB agar, sheep blood agar, chocolate agar (Nippon Becton Dickinson Co. Ltd., Tokyo, Japan), brucella HK agar (Kyokuto Pharmaceutical Co. Ltd., Tokyo, Japan), and GAM (Gifu Anaerobic Medium) agar (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). BTB agar was incubated in the aerobic chamber at 37°C, sheep blood agar and chocolate agar were incubated in the O₂/CO₂ incubator, and brucella HK agar was incubated anaerobic chamber for 48 hours, respectively. GAM agar was incubated in the ambient chamber at 37°C for 72 hours. When the colony forming was detected, it was considered to be positive for bacterial translocation.

Statistical analysis. The results are expressed as mean $\pm$ SEM. The Mann-Whitney *U* test and chi-square test were used to evaluate differences between two groups. A *P* value <0.05 was considered statistically significant.

RESULTS

Expression of TLR2 mRNA and protein. In SAP group, expression of TLR2 mRNA began to decrease 3 hours after the induction, and was significantly lower than Sham group 6 hours after the induction (Fig. 1A). The initial concentration rate of mRNA was 0.77 ± 0.12 in SAP group. Expression of TLR2 protein did not change 3 hours after induction of SAP, but decreased 6 hours after induction of SAP compared with sham group (Fig. 1B).

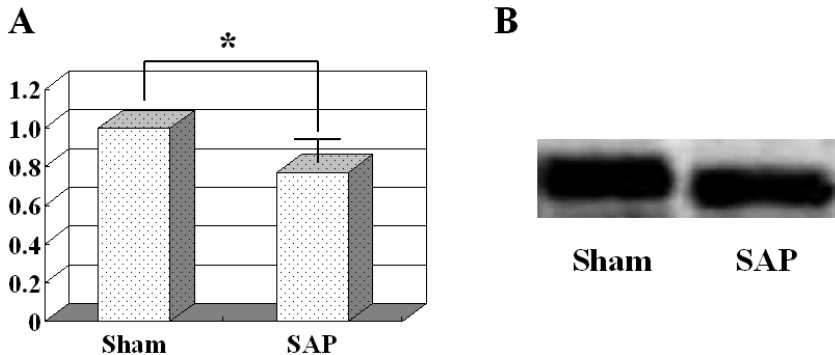


FIG. 1. A, Expression of Toll-like receptor (TLR) 2 mRNA on macrophages in severe acute pancreatitis (SAP). TLR2 mRNA 6 hours after induction of SAP was analyzed by real-time RT-PCR. * $P < 0.05$ between two groups. B, Expression of TLR2 protein on macrophages in SAP. TLR2 protein 6 hours after induction of SAP was analyzed by western blotting. Results shown are representative of three independent experiments. 50 μ g of protein was applied in each lane.

TLR2-mediated TNF- α production. LTA-induced TNF- α release from macrophages was significantly depressed in SAP group compared with sham group at concentrations of 10^2 and 10^3 ng/ml (Fig. 2).

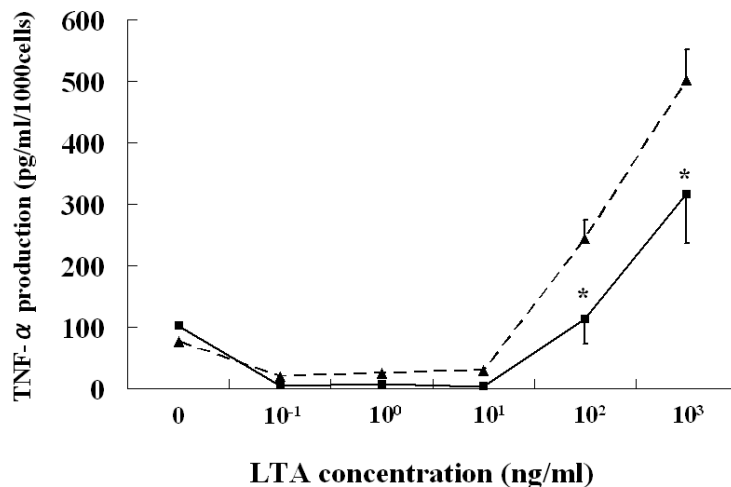


FIG. 2. TLR2-mediated TNF- α production by macrophages in SAP. Macrophages were stimulated with lipoteichoic acid (LTA) (0 – 10^3 ng/ml) for 4 hours. TNF- α concentrations of culture medium were determined by ELISA. $\blacktriangle$, Sham; $\blacksquare$, SAP. * $P < 0.05$ vs. Sham.

Expression of TLR4 mRNA and protein. In SAP group, expression of TLR4 mRNA began to decrease 3 hours after the induction, and was significantly lower than Sham group 6 hours after the induction (Fig. 3A). The initial concentration rate of mRNA was 0.42 ± 0.18 in SAP group. Expression of TLR4 protein did not change 3 hours after induction of SAP, but decreased 6 hours after induction of SAP compared with sham group (Fig. 3B).

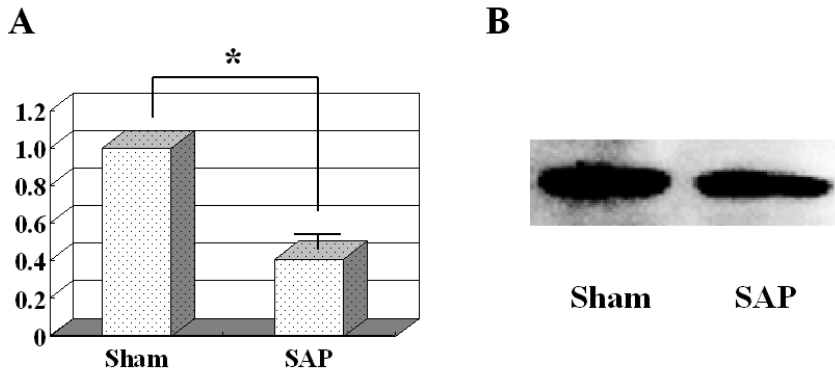


FIG. 3. A, Expression of TLR4 mRNA on macrophages in SAP. TLR4 mRNA 6 hours after induction of SAP was analyzed by real-time RT-PCR. * $P < 0.05$ between two groups. B, Expression of TLR4 protein on macrophages in SAP. TLR4 protein 6 hours after induction of SAP was analyzed by western blotting. Results shown are representative of three independent experiments. 50 μ g of protein was applied in each lane.

TLR4-mediated TNF- α production. LPS-induced TNF- α release from macrophages was significantly depressed in SAP group compared with sham group at concentrations of 10^3 , 10^4 and 10^5 ng/ml (Fig. 4).

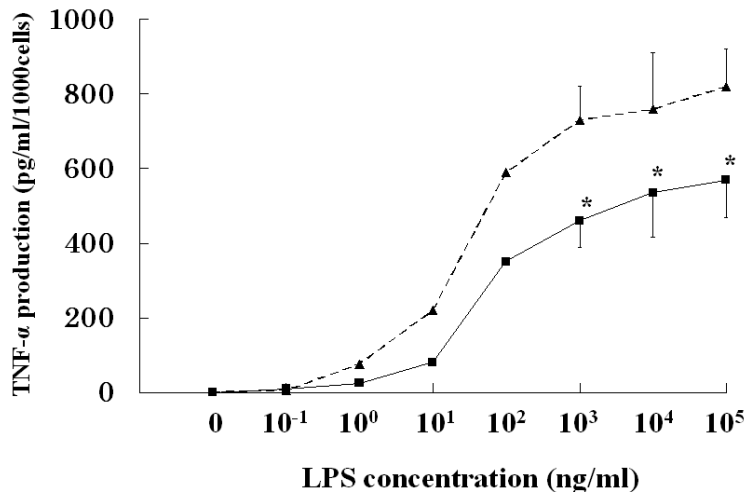


FIG. 4. TLR4-mediated TNF- α production by macrophages in SAP. Macrophages were stimulated with lipopolysaccharide (LPS) (0 – 10^5 ng/ml) for 4 hours. TNF- α concentrations of culture medium were determined by ELISA. $\blacktriangle$, Sham; $\blacksquare$, SAP. * $P < 0.05$ vs. Sham.

Endotoxin/bacterial translocation in this model. Blood endotoxin levels were not detected 0, 6 and 12 hours after induction of SAP, but were significantly elevated (90 ± 20 pg/ml) 18 hours after induction of SAP (Table). Positive rate of bacterial culture of MLNs was 0% (0/10) 0, 6, and 12 hours after induction of SAP, but it was significantly increased to 50% (6/12) 18 hours after induction of SAP (Table).

TABLE. Endotoxin/bacterial translocation in this model

Time after induction of pancreatitis (hour)	Blood endotoxin level	Positive rate of bacterial culture of mesenteric lymph nodes
0	Not detected (<1.0 pg/ml)	0% (0/10)
6	Not detected (<1.0 pg/ml)	0% (0/10)
12	Not detected (<1.0 pg/ml)	0% (0/10)
18	90 ± 20 pg/ml *	50% (6/12) *

* $P < 0.05$ vs. 0, 6, and 12-hour groups

DISCUSSION

TLRs play a crucial role in host defense against microbial infection. TLR2 and TLR4 on macrophages represent the main sensors to recognize molecular products from gram-positive and gram-negative bacteria, respectively, as innate immune system. We used here alveolar macrophages as representative of macrophages, because peritoneal macrophages are perished by cytotoxic effect of pancreatitis-associated ascitic fluid and peripheral blood monocytes cannot be obtained enough to accomplish our all experiments. In this study, we have first demonstrated that expression of TLR2 and TLR4 on macrophages was down-regulated in the early phase of SAP, and that agonist-induced cytokine production was also suppressed. These results suggest that the impaired responsiveness to the agonists is derived from decreased expression of TLRs. This suppression of immune response from the early phase may be implicated in the mechanism of subsequent infectious complications.

There have been several reports concerning TLRs in acute pancreatitis. Li *et al.* demonstrated that TLR4 was detected in exocrine pancreas and was up-regulated in the early stage of rat mild acute pancreatitis (11). It was reported that the expression of TLR2 and TLR4 mRNA was increased in lung and liver, and that the up-regulation was related to lung and liver injuries in rat SAP (29,31). From these observations, it is possible that TLRs may be up-regulated in various tissues in the early phase of SAP and that the up-regulation facilitates the inflammatory response and organ injuries. However, expression of TLRs on macrophages or function of macrophages via TLRs has not yet been clarified in SAP.

The mechanism for decreased expression of TLR2 and TLR4 on macrophages in SAP remains to be elucidated. Expression of TLRs is modulated by various factors such as microbial invasion, microbial components, and cytokines. Because SAP is noninfectious (sterile) inflammation in the early stage, participation of cytokine is thought. Recent studies have shown that macrophage migration inhibitory factor, interferon-gamma, and IL-2 up-regulate the expression of TLRs (3,14,19), and that colony-stimulating factor and IL-4 down-regulate the expression (14,23). External stresses, such as surgery, trauma, and burns, induce the production of not only proinflammatory cytokines but also anti-inflammatory cytokines (IL-4 and IL-10) (6). Anti-inflammatory cytokines suppress immune responses and increase the risk of infection. Moreover, it is possible that immunosuppressive factors (catecholamines and steroids) induced by the stresses may also down-regulate the expression

of TLRs. Therefore, it is speculated that these anti-inflammatory cytokines or immunosuppressive factors may down-regulate the expression of TLR2 and TLR4 in SAP.

Recently, two investigations have been performed to clarify the role of TLR4 in SAP or septic condition using wild-type and TLR4-deficient mice. Pastor et al. reported that LPS did not worsen the pancreatitis in wild-type and TLR4-deficient mice, but that LPS worsened pancreatitis-associated lung injury in only wild-type mice (17). Van Westerloo et al. reported that TLR4 deficiency did not influence the severity of pancreatitis but that TLR4 deficiency resulted in a reduced early release of proinflammatory cytokines and an increased bacterial load during abdominal sepsis (26). These results suggest that TLR4 is closely related to the induction of organ injury and the defense of bacterial infection.

It is generally reported that TLRs are up-regulated in inflammatory conditions (7,15) and that TLRs are down-regulated in immunosuppressive conditions (9). Ikushima et al. reported that expressions of TLR2 and TLR4 on peripheral blood mononuclear cells were down-regulated after gastrointestinal surgery and that agonist-induced TNF- α production was decreased (9). Therefore also in SAP, it is postulated that up-regulation of TLRs may facilitate the inflammatory response and function protectively against infection, and that down-regulation of TLRs may suppress the inflammation and facilitate the subsequent infection (E/BT). In our results, expression of TLR2 and TLR4 on macrophages was diminished 6 hours after induction of SAP accompanied with the depression of cytokine production, indicating the suppression of innate immune system. Indeed in our model, after the decrease of TLR2 and TLR4 expression, E/BT occurred 18 hours after induction of SAP. We could not demonstrate the direct evidence for relationship between decreased expression of TLRs and E/BT, but it is generally accepted that TLR2^{-/-} or TLR4^{-/-} mice have an increased susceptibility to infections (5,10,22,26,28). Moreover, we have recently found that gram-negative bacterial translocation is significantly increased in TLR4-deficient mice SAP compared with wild-type mice SAP (21). Therefore, it is speculated that down-regulation of TLR2 and TLR4 on macrophages may be implicated in the mechanism of E/BT. Further investigations are necessary to elucidate the role of TLRs and TLR signaling in the host defense mechanism in SAP.

ACKNOWLEDGEMENTS

This investigation was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and Grant-in-Aid for Scientific Research from the Ministry of Health, Labor and Welfare of Japan.

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