Lipopolysaccharide triggered TNF-α-induced hepatocyte apoptosis in a murine non-alcoholic steatohepatitis model

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Background/Aims: Endogenous gut-derived bacterial endotoxins have been implicated as an important cofactor in the pathogenesis of liver injury, although their contribution to the progression of non-alcoholic steatohepatitis (NASH) remains unclear.

Methods: Male C57BL/6 mice were fed a methionine–choline-deficient (MCD) diet or a standard diet for 17 days, following which they were injected with lipopolysaccharide (LPS) intraperitoneally and sacrificed after 6 h. In an in vitro experiment, RAW264.7 cells, a mouse macrophage cell line, and primary mouse hepatocytes were co-treated with hydrogen peroxide (H2O2) and LPS or tumour necrosis factor (TNF)-α.

Results: Compared to the control mice, LPS treatment significantly increased hepatic TNF-α production in MCD mice. LPS also significantly increased TUNEL-positive cells, which were especially observed in the perivenular area. The apoptotic change was inhibited by co-treatment with a neutralizing anti-mouse TNF receptor antibody or pentoxifylline. In an in vitro experiment, treatment with H2O2 synergistically enhanced LPS-induced TNF-α production in RAW264.7 cells, accompanied by an up-regulation of CD14 mRNA. Moreover, co-treatment with TNF-α and H2O2-induced apoptosis in primary hepatocytes, although neither TNF-α nor H2O2 could do so independently.

Conclusions: LPS up-regulated TNF-α production, which induced hepatocyte apoptosis in a murine NASH model. LPS may play a key role in the pathogenesis of NASH.

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a major form of chronic liver disease in adults and children [1]. Non-alcoholic steatohepatitis (NASH) is characterized by steatosis, inflammation, and progressive fibrosis and can ultimately lead to cirrhosis and end-stage liver disease [2]. NASH is observed in a subset of NAFLD patients, although the exact mechanisms leading NAFLD to NASH and cirrhosis remain poorly understood.

The histological characteristics of NASH resemble those of alcoholic steatohepatitis (ASH), suggesting that both diseases may have a similar pathogenesis [3,4]. Studies in a rodent ASH model showed that ethanol
consumption increases both the permeability of the small intestine and endotoxins, which modulate alcohol-induced liver damage. Intestinal bacteria, bacterial endotoxins and the endotoxin-inducible proinflammatory cytokines, such as tumour necrosis factor (TNF)-α, incite necroinflammatory changes in steatohepatitis and hepatic fibrosis [5–7]. A previous report showed that small intestinal bacterial overgrowth occurred significantly more often in NASH patients than in control subjects [8]. Furthermore, administration of probiotics can improve liver damage and functional tests of both human subjects and ob/ob mice affected by NASH [9,10]. These findings gave rise to the hypothesis that gut-derived bacterial endotoxins could be involved in triggering liver injury in not only ASH but also NASH. However, a relationship between endotoxins and the pathology of NASH has not yet been established. To test our hypothesis, we evaluated the effect of lipopolysaccharide (LPS) in a dietary NASH model induced by a methionine–choline-deficient (MCD) diet.

2. Materials and methods

2.1. Animals and experimental protocol

Male C57BL/6 mice of 8 weeks of age were purchased from Sankyo Labo Service Corporation Inc. (Tokyo, Japan). They were housed in a light-, temperature- and humidity-controlled room. Initially, all mice were fed standard laboratory food during a 2-week acclimation period. We prepared the MCD diet, which contained corn oil and sucrose [40% (w/w) fat and 40% (w/w) carbohydrate]. When the mice reached the age of 10 weeks, they were fed the MCD or standard diet for 17 days. They were then injected intraperitoneally with LPS from E. coli (0.5 mg/kg) twice per week from 8th day and sacrificed after 6 h to obtain serum and liver tissue. Pentoxifylline (10%) and embedded in paraffin. Liver tissue sections were stained by hematoxylin and eosin (H–E) staining and Sirius red staining. (10%) and embedded in paraffin. Liver tissue sections were stained by hematoxylin and eosin (H–E) staining and Sirius red staining.

2.2. Histological analysis

Liver specimens were fixed overnight in buffered formaldehyde (10%) and embedded in paraffin. Liver tissue sections were stained by hematoxylin and eosin (H–E) staining and Sirius red staining. The fibrotic areas were measured in three sections per mouse using an image analyzing system (VH analyzer; KEYENCE, Osaka, Japan).

2.3. Immunohistochemistry

Formalin-fixed and paraffin-embedded sections of the liver were used in this study. A rat monoclonal antibody against F4/80 (dilution 1:500; Serotec, Oxford, UK), a mouse monoclonal antibody against 8-hydroxy-2-deoxyguanosine (8-OHdG; dilution 1:10; Nikken SEI Co., Ltd., Sizuoka, Japan), a rabbit polyclonal antibody against mouse CD14 (dilution 1:5000; biometec, Greifswald, Germany), or a mouse monoclonal antibody against α-smooth muscle actin (α-SMA; dilution 1:100; Dako, Tokyo, Japan) was applied to the sections. The sections were incubated with secondary antibodies, and the immunoreactive products were visualized using a DAB reagent and counterstained with hematoxylin.

2.4. Measurement of thioarbituric acid-reactive substances and plasma TNF-α

Hepatic thioarbituric acid-reactive substances (TBARS), considered to be oxidative stress markers, were measured using the Lab Assay™ TBARS kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer’s instructions. Plasma concentrations of mouse TNF-α were measured by ELISA using a Quantikine kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

2.5. Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from mouse livers using the RNeasy mini kit (QIAGEN, Tokyo, Japan). Approximately 1 μg of total RNA was converted into cDNA using the QuantiTect Reverse Transcription kit (QIAGEN, Tokyo, Japan). Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using the ABI 7300 sequence detection system (Applied Biosystems, Foster City, CA). Primers used for TNF-α were: forward 5'-CTGGGACACGTGACCTGACT-3', reverse 5'-GCCATCCAGGGAAGGTCTG-3', CD14: forward 5'-GGCTTTTCGAGCCTATCT-3', reverse 5'-TGACCTGGATCTGAAGATG-3'. A multiplex comparative threshold cycle (Ct) method was used in which the Ct value reflects the cycle number at which the DNA amplification is first detected. All PCRs were carried out in duplicate.

2.6. TUNEL staining

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) was performed using the ApopTag Plus peroxidase in situ apoptosis detection kit (CHEMICON, Billerica, MA) according to the manufacturer’s instructions. In brief, paraffin sections were digested with 20 μg/mL of proteinase K (TaKaRa, Shiga, Japan) for 15 min at room temperature and reacted with terminal deoxynucleotidyl transferase enzyme for 60 min at 37 °C. The sections were then incubated with anti-digoxigenin conjugate at room temperature for 30 min, followed by incubation with diamonsobenzide solution.

2.7. Caspase-3 activity

Caspase-3 was measured using a Caspase-3/3′CPP32 colorimetric assay kit (Biovision, Mountain View, CA) according to the manufacturer’s instructions. Absorbance was measured at 405 nm.

2.8. Western blot analysis

Proteins from homogenized liver tissue were analyzed by Western blotting. The membrane was exposed to specific polyclonal antibodies against apoptosis signal-regulating kinase 1 (ASK1; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-ASK1, c-Jun N-terminal kinase (JNK; Cell Signaling Technology, Beverly, MA) and phospho-JNK (New England Biolabs, Beverly, MA). The membrane was washed, exposed to alkaline phosphatase-conjugated secondary antibodies, and visualized by incubation in CDP star assay buffer (New England Biolabs) according to the company’s protocol.

2.9. Cell culture

Primary hepatocytes were isolated from C57BL/6 mice as reported previously [11]. The murine monocyte/macrophage cell line RAW 264.7 was obtained from ATCC (Rockville, MD). The cells were cultured at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium (ASAHI...
TECHNO GLASS Co., Tokyo, Japan), and supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin plus 10% foetal bovine serum.

2.10. Quantification of apoptosis induced by H$_2$O$_2$ and TNF-α

The Cell Death Detection ELISA Plus assay kit (Roche Applied Science, Mannheim, Germany) was used to detect the presence of nucleosomes in the cytoplasm, which is a well-known hallmark of apoptosis. A total of 25 x 10$^3$ primary hepatocytes per well were plated in duplicate on a 48-well collagen-coated plate and incubated overnight. Then, the cells were treated with 0, 20 or 100 µM H$_2$O$_2$, and 20 ng/mL TNF-α was added 5 min after H$_2$O$_2$ treatment. After incubation for 16 h, the samples were assayed according to the manufacturer’s instructions. DNA fragmentation was detected using the apoptotic DNA ladder kit (Roche Applied Science, Mannheim, Germany).

2.11. Statistical analysis

Data are presented as means ± SEM and analyzed statistically using one-way ANOVA followed by Fisher’s protected least-significance difference test or the Mann–Whitney U test. p < .05 was considered statistically significant.

3. Results

Liver histology showed steatohepatitis with fatty droplets after 17 days of MCD diet feeding with LPS injection (Fig. 1A). Serum ALT level of the MCD diet-fed mice was significantly higher than that of the control mice (Fig. 1B). To assess hepatic oxidative stress, we examined the expression of 8-OHdG and TBARS. The expression of 8-OHdG, a reliable marker of oxidative DNA damage, was clearly detected in the nucleus of hepatocytes in MCD mice (Fig. 1C). However, it was not detected in control mice. TBARS were also significantly higher in the MCD diet-fed mice than in the control mice (Fig. 1D). On the other hand, a single dose of LPS did not affect liver histology, plasma ALT level, or TBARS expression (Fig. 1A, B and D). Similarly, the number of 8-OHdG-positive cells was not affected by LPS (data not shown).

The MCD diet caused hepatic inflammation with fatty droplets. Compared to the control mice, LPS treatment dramatically up-regulated hepatic mRNA expression and the serum level of TNF-α in the MCD diet-fed mice (Fig. 2A and B). To identify the inflammatory cell types, we performed immunohistochemical analysis using a monoclonal antibody specific for F4/80 antigen, a surface marker of mouse monocytes/macrophages and Kupffer cells. F4/80-positive cells were more prevalent in the MCD diet-fed mice (Fig. 2C). Moreover, compared to the control mice, the MCD diet significantly increased hepatic CD14 mRNA and protein expression, a functional LPS receptor on macrophages, in mice (Fig. 2D and E). Immunohistochemical study showed many activated macrophages stained with anti-CD14 antibody in the MCD diet-fed mice (Fig. 2F). Taken together, these observations suggest that hepatic susceptibility to LPS was up-regulated by the increased CD14 expression in the liver of the MCD diet-fed mice; thus, LPS dramatically increased TNF-α production.
To assess hepatocyte apoptosis, we performed the TUNEL assay on paraffin sections. LPS treatment induced a significant increase in the number of TUNEL-positive cells in the liver (Fig. 3 A). The number of TUNEL-positive cells induced by LPS was significantly higher in the MCD diet-fed mice than in the control mice (Fig. 3 B). This was especially observed in the perivenular area (zone 3). We also assessed caspase-3 activity in the liver. LPS increased the activation of caspase-3 in the MCD diet-fed mice compared to the control mice, but not significantly (0.23 ± 0.036 vs. 0.19 ± 0.015 mU, \( p = .087 \)). Pretreatment with PTX, an inhibitor of TNF-\( \alpha \) production [12], down-regulated TNF-\( \alpha \) production induced by LPS in the MCD diet-fed mice (217.2 ± 9.2 vs. 331 ± 82.8, \( p < .05 \)). Furthermore, PTX and neutralizing TNFR Ab significantly decreased the number of TUNEL-positive cells, with the latter blocked more effectively (Fig. 3 B). These results suggest that LPS-induced hepatocyte apoptosis was mediated by TNF-\( \alpha \).

To assess the effect of reactive oxygen species (ROS) on LPS-induced TNF-\( \alpha \) production by Kupffer cells, we incubated RAW264.7 cells with LPS and \( \mathrm{H}_2\mathrm{O}_2 \). TNF-\( \alpha \) production from RAW264.7 cells was up-regulated by LPS in a dose-dependent manner (Fig. 4 A). Likewise, the production of TNF-\( \alpha \) was up-regulated by \( \mathrm{H}_2\mathrm{O}_2 \) with a significant effect at more than 100 \( \mu \mathrm{M} \) compared with untreated cells (Fig. 4 B). Interestingly, co-treatment using 50 \( \mu \mathrm{M} \) \( \mathrm{H}_2\mathrm{O}_2 \) with LPS, which was not a significantly effective dose, enhanced TNF-\( \alpha \) production compared to treatment with LPS alone, which was inhibited by \( \mathrm{N} \)-acetyl-L-cysteine (NAC) (Fig. 4 C). We also showed that \( \mathrm{H}_2\mathrm{O}_2 \) increased the CD14 mRNA expression in RAW264.7 cells in a dose-dependent manner, and the expression was inhibited by NAC (Fig. 4 D). These results suggest that ROS not only up-regulated TNF-\( \alpha \) production but also increased the susceptibility of macrophages to LPS by increasing CD14 expression.

To access the participation of ROS and TNF-\( \alpha \) in hepatocyte apoptosis, primary cultured hepatocytes were incubated with \( \mathrm{H}_2\mathrm{O}_2 \) and TNF-\( \alpha \). Neither \( \mathrm{H}_2\mathrm{O}_2 \) nor TNF-\( \alpha \) treatment produced a significant increase in hepatocyte apoptosis individually, although the latter
had slightly enhanced effects. However, pretreatment with H$_2$O$_2$ synergistically enhanced TNF-$\alpha$-induced hepatocyte apoptosis compared with untreated cells. In addition, pretreatment with NAC significantly suppressed apoptosis induced by co-treatment with H$_2$O$_2$ and TNF-$\alpha$ (Fig. 5A). We also confirmed that the

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**Fig. 4.** LPS-induced TNF-$\alpha$ production was enhanced by H$_2$O$_2$. TNF-$\alpha$ concentration of the medium was assessed by ELISA after 6 h of treatment. (A) LPS- and (B) H$_2$O$_2$-induced TNF-$\alpha$ production from RAW 264.7 cells in a dose-dependent manner. (C) LPS-induced TNF-$\alpha$ production was additively increased by pretreatment with H$_2$O$_2$ for 5 min, which was inhibited by pretreatment with NAC for 30 min. (D) CD14 mRNA expressions of RAW264.7 cells. Values are expressed as means ± SEM. ANOVA, *$p < .05$ vs. untreated cells, /$p < .05$ vs. H$_2$O$_2$ treatment alone, #$p < .05$.**

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**Fig. 5.** H$_2$O$_2$ enhanced TNF-$\alpha$-induced hepatocyte apoptosis by activating ASK1 and JNK. Primary hepatocytes were pretreated with H$_2$O$_2$ (0, 20, 100 $\mu$M) for 5 min before stimulation with TNF-$\alpha$ (20 ng/mL). NAC (1, 10 mM) was added for 30 min before H$_2$O$_2$ treatment. Apoptosis was assessed using a cell death detection assay and DNA fragmentation after 16 h of incubation. (A) H$_2$O$_2$ facilitated TNF-$\alpha$-mediated hepatocyte apoptosis. Accordingly, NAC inhibited apoptosis in a dose-dependent manner. (B) DNA fragmentation of hepatocytes was observed by treatment with H$_2$O$_2$ (100 $\mu$M) and TNF-$\alpha$. (C and D) Representative Western blot analysis of ASK1 and JNK. At 30 min after TNF-$\alpha$ treatment, ASK1 phosphorylation was enhanced by H$_2$O$_2$ and phosphorylation was inhibited by NAC. (D) JNK phosphorylation at the indicated time after treatment. Values are expressed as means ± SEM. ANOVA, *$p < .05$. PC, positive control.
DNA ladder of hepatocytes was induced by co-treatment, which was inhibited by NAC (Fig. 5B). These findings indicated that ROS enhanced TNF-α-induced hepatocyte apoptosis.

To determine the involvement of the ASK1–JNK signal pathway in TNF-α-induced hepatocyte apoptosis, we examined the activity of ASK1 and JNK in cultured hepatocytes by Western blot analysis. TNF-α treatment induced ASK1 activation in hepatocytes and co-treatment with H₂O₂ further enhanced ASK1 activation, which was suppressed by NAC (Fig. 5C). Next, JNK activity was studied in hepatocytes at different time points. JNK activity was increased at 30 min and decreased at 60 min by single treatment of TNF-α. However, co-treatment with H₂O₂ sustained its activation until 120 min; meanwhile, NAC inhibited JNK activation (Fig. 5D). These results suggest that the ASK1–JNK signal pathway was enhanced by ROS and may be involved in the TNF-α-induced hepatocyte apoptosis in NASH.

To examine the progression of the present NASH model, mice were injected with LPS or saline twice per week for 3 weeks. In MCD mouse livers, multiple LPS treatments induced fibrosis as detected by Sirius red staining, and the fibers extended from the central vein (Fig. 6A and B). Immunostaining examination showed the appearance of increased numbers of α-SMA-positive cells in zone 3 (Fig. 6C), which were thought to be activated hepatic stellate cells. These pathological findings were similar to those observed in the fibrosis occurring in human NASH.

4. Discussion

In this study, we showed that LPS up-regulated TNF-α production and facilitated hepatocyte apoptosis in a murine NASH model. Furthermore, an in vitro study showed that ROS increased the susceptibility of macrophages to LPS and sensitized hepatocytes to TNF-α-induced apoptosis. This is the first study to report that LPS plays an important role in TNF-α-induced hepatocyte apoptosis in a NASH model.

First, we injected LPS intraperitoneally into the MCD diet-fed mice, which serve as a nutritional model of NASH. LPS significantly increased hepatic TNF-α production in the MCD diet-fed mice. Likewise, patients with NASH have a higher prevalence of small intestinal bacterial overgrowth and higher TNF-α levels than control subjects [8]. Within the liver, Kupffer cells are major sources of TNF-α produced in response to LPS [13]. The process by which LPS activates Kupffer cells seems to be mediated by the LPS-binding protein, CD14 and Toll-like receptor 4 [13]. We focused on the CD14 expression in the MCD diet-fed mice because it was reported that the promoter polymorphism of CD14 is a risk factor for ASH and NASH [14,15]. The CD14 expression of Kupffer cells, which is low in normal human liver, increases in inflammatory liver diseases [16]. Haziot et al. reported that TNF-α production was decreased in genetically engineered CD14-deficient mice by downregulating sensitivity to LPS [17]. In contrast, the CD14 transgenic mice that overexpress CD14 on monocytes have increased sensitivity to LPS [18]. These changes in CD14 expression could be the underlying mechanism that regulates the liver’s sensitivity to LPS toxicity. Rivera et al. reported that CD14 mRNA expression and sensitivity to LPS was up-regulated in the liver of mice fed an MCD diet [19,20]. Their reports were coincident with our present study. Furthermore, we also showed in an in vitro experiment that H₂O₂ stimulated CD14 mRNA expression, which was followed by LPS-
induced TNF-α production of macrophages. Taken together, these results suggest that ROS up-regulated CD14 expression, which increased the susceptibility of Kupffer cells to LPS. As a result, LPS-induced TNF-α production is thought to be up-regulated in NASH.

Second, we showed that LPS treatment of the MCD diet-fed mice increased hepatocyte apoptosis, whereas PTX and TNFR Ab inhibited apoptosis. A recent study showed that hepatocyte apoptosis is significantly elevated in patients with NASH and is correlated with disease severity [21]. It was also reported that the cytokeratin-18 fragment, a specific label of early apoptotic cells, is a strong and independent predictor of NASH in humans [22]. These results suggest that hepatocyte apoptosis plays an important role in the development of NASH. Our results indicate that TNF-α is associated with LPS-induced hepatocyte apoptosis in mice fed an MCD diet. TNF-α initiates accumulation of ROS by binding the TNF receptor 1, which promotes cell death through activation of the JNK MAP kinase (MAPK) cascade [23]. However, it has been reported that hepatocytes, like most untransformed cells, are resistant to TNF-α [23–26]. Actually, TNF-α is well tolerated by primary cultured hepatocytes and by the liver when injected exogenously into healthy rats [27]. Similarly, in the present study, TNF-α treatment of cultured hepatocytes without pretreatment did not induce apoptosis in vitro. Interestingly, pretreatment with H2O2 significantly enhanced TNF-α-induced hepatocyte apoptosis. Furthermore, NAC inhibited hepatocyte apoptosis induced by co-treatment with TNF-α and H2O2. These results suggested that ROS accumulation enhanced TNF-α-induced hepatocyte apoptosis. Although the precise mechanisms remain to be determined, ROS may play an important role in TNF-α-induced hepatocyte apoptosis in NASH.

Hepatocyte apoptosis and fibrosis was localized predominantly in zone 3, where histological damage is often observed in humans with NASH [28]. It was reported that apoptosis can activate stellate cells by two recognized mechanisms involving phagocytosis of apoptotic bodies. Both Kupffer and stellate cell engulfments of apoptotic bodies result in expression of transforming growth factor β, a profibrogenic cytokine that activates stellate cells by paracrine and autocrine mechanisms, respectively [29–31]. Although the precise mechanisms of hepatic fibrosis remain unclear, TNF-α-induced hepatocyte apoptosis by LPS may be associated with the progression of fibrosis in human NASH.

Third, we observed that H2O2 facilitated TNF-α-induced hepatocyte apoptosis with activated ASK1 and sustained JNK activation in vitro. ASK1 is a ubiquitously expressed MAPK kinase that plays an important role in apoptosis [32]. Overexpression of wild-type or constitutively active ASK1 induces apoptosis in various cells through mitochondria-dependent caspase activation [33]. A recent study showed that ROS promote TNF-α-induced cell death with sustained JNK activation in murine embryonic fibroblasts [34]. TNF-α- and H2O2-induced sustained activation of JNK is lost in ASK1 knockout embryonic fibroblasts, and these cells are resistant to TNF-α- and H2O2-induced apoptosis [32]. Similarly, in our study, H2O2 facilitated hepatocyte apoptosis together with activated ASK1 and sustained JNK activation. However, Kamata et al. reported that the inhibition of MAPK phosphatase by ROS was important for TNF-α-induced apoptosis in murine embryonic fibroblasts [35]. The difference in these results regarding the association between TNF-α-induced apoptosis and ROS is not entirely clear. Further investigations are needed to clarify the precise signal pathway, which will determine the role of ASK1 in hepatocyte apoptosis in NASH patients.

In summary, we showed that LPS up-regulated TNF-α production by increasing susceptibility of Kupffer cells and facilitated TNF-α-mediated hepatocyte apoptosis in a murine NASH model. Although further studies are needed to identify the precise mechanisms of hepatocyte apoptosis in NASH, these concepts merit further investigation of the mechanism contributing to the development of cirrhosis in NASH and potential therapeutic strategies for these diseases.

References


