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Full paper

Recombinant human soluble thrombomodulin improved lipopolysaccharide/D-galactosamine-induced acute liver failure in mice

Wataru Osumi [a], Denan Jin [b], Yoshiro Imai [a], Keitaro Tashiro [a], Zhong-Lian Li [c], Yoshinori Otsuki [c], Kentaro Maemura [c], Koji Komeda [a], Fumitoshi Hirokawa [a], Michihiro Hayashi [a], Shinji Takai [d,*], Kazuhisa Uchiyama [a]

[a] Department of General and Gastroenterological Surgery, Osaka Medical College, Takatsuki 569-8686, Japan
[b] Department of Pharmacology, Osaka Medical College, Takatsuki 569-8686, Japan
[c] Department of Anatomy and Cell Biology, Osaka Medical College, Takatsuki 569-8686, Japan
[d] Laboratory for Innovative Medicine, Graduate School of Medicine, Osaka Medical College, Takatsuki 569-8686, Japan

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ABSTRACT

The effect of recombinant human soluble thrombomodulin (TM-α) on acute liver failure (ALF) is unclear, and we elucidated the effect of TM-α in lipopolysaccharide (LPS)/D-galactosamine (GalN)-induced ALF in mice. Placebo (saline) or TM-α (100 mg/kg) was administered 1 h after LPS/GalN administration. Survival rates were evaluated for 24 h after LPS/GalN administration. Plasma and liver samples were evaluated 1, 3, and 7 h after LPS/GalN administration. Survival rates were significantly higher in the TM-α-treated group than in the placebo group. A significant augmentation of plasma high-mobility group box 1 protein (HMGB1) was observed 7 h after LPS/GalN administration. In the TM-α-treated mice, plasma HMGB1 was significantly lower than in the placebo group. A significant augmentation of hepatic nuclear factor (NF)-κB p65 was observed in the placebo-treated group, whereas a significant reduction, relative to placebo, was observed in the TM-α-treated group. Hepatic expression of tumor necrosis factor (TNF)-α and myeloperoxidase were significantly increased in the placebo group, and were similarly significantly attenuated in the TM-α-treated group. TM-α treatment also produced a significant attenuation of liver neutrophil accumulation after LPS/GalN administration. Thus, TM-α may become a useful treatment strategy for reducing the symptoms of ALF via the attenuation of LPS/GalN-induced HMGB1 levels.

1. Introduction

Acute liver failure (ALF) is a characterized by a rapid loss of hepatocyte function, and its major causes are viral infection, autoimmune hepatitis, and drug allergy (1). ALF is associated with high mortality in patients, and liver transplantation is the only effective therapeutic strategy for attenuating ALF. ALF is typically initiated by the activation of inflammatory cells such as macrophages, which release several inflammatory cytokines, resulting in massive death of parenchymal hepatocytes (2).

Combined administration of lipopolysaccharide (LPS) and D-galactosamine (GalN) has become an established method for inducing ALF in experimental animals (3,4). LPS causes sinusoidal injury and intrahepatic fibrin deposition, which results in severe liver dysfunction (5). GalN potentiates the toxic effects of LPS in the liver through upregulation of toll-like receptor (TLR)-4 mRNA expression. TLR-4 specifically binds LPS in the liver (6). GalN-potentiated binding of LPS to TLR-4 in the liver promotes the activation of nuclear factor (NF)-κB, ultimately inducing the production of proinflammatory cytokines such as tumor necrosis factor (TNF)-α (7). Activation of NF-κB induces inflammation and apoptosis in the liver and increases TNF-α production, which exacerbates the inflammation (3). Consequently, the combined administration of LPS and GalN has been shown to specifically induce liver injury via activation of NF-κB and increased production of TNF-α (8).

* Corresponding author. Laboratory for Innovative Medicine, Graduate School of Medicine, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki 569-8686, Japan. Tel.: +81 72 684 6021; fax: +81 72 684 6730.
E-mail address: pha010@art.osaka-med.ac.jp (S. Takai).
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High-mobility group box 1 (HMGB1) is an intra-nuclear non-histone DNA-binding protein that has both nuclear and extracellular functions (9). HMGB1 is secreted by necrotic and damaged cells, and release of HMGB1 into the extracellular space induces the production of proinflammatory cytokines such as TNF-α, resulting in a vicious cycle in which activated cytokines induce further release of HMGB1 (10,11). Upregulation of HMGB1 has been shown to be a lethal late-phase mediator of sepsis (12).

Thrombomodulin (TM) is a thrombin receptor on the surface of endothelial cells and plays a crucial role in regulating coagulation (13). Additionally, TM activates protein C, resulting in anti-inflammatory effects such as the suppression of TNF-α via inhibition of macrophage activation (14). In the present study, we used recombinant human soluble thrombomodulin (TM-α), which not only binds to HMGB1 but also cleaves HMGB1 by forming a complex with thrombin, resulting in attenuation of HMGB1-mediated inflammation (15). TM-α has proven to be useful in treating disseminated intravascular coagulation in clinic. In a rat LPS-induced sepsis model, TM-α was shown to significantly attenuate the upregulation of plasma HMGB1 levels (16). However, the role of TM-α in ALF remains unclear. In the present study, we evaluated the effect of TM-α on survival rate in a mouse model of LPS/GalN-induced ALF.

2. Materials and methods

2.1. Drugs

LPS (*Escherichia coli*, 0111:B4) and GalN were purchased from Sigma (St. Louis, MO). TM-α was obtained from Asahi Kasei Pharma Co. Ltd. (Tokyo, Japan).

2.2. Animal model

Eight-week-old male C57BL/6 mice (n = 84) were obtained from Japan SLC (Shizuoka, Japan) and housed in a temperature-, humidity-, and light-controlled room. The effect of TM-α on the survival rate over the 24 h period following administration (i.p.) of LPS (4 μg/kg)/GalN (600 mg/kg) was evaluated by administering (s.c.) TM-α (100 mg/kg) or placebo (saline) 1 h after LPS/GalN injection (each group: n = 20). Measurements of plasma and liver parameters were taken before and 1, 3, and 7 h after LPS/GalN injection (n = 6) to assess time-dependent changes. The effects of TM-α were evaluated following administration (s.c.) of TM-α (100 mg/kg) or placebo (saline) 1 h after LPS/GalN injection, and blood and liver tissue were obtained at 7 h after LPS/GalN injection (n = 8). Age-matched normal mice were used as a control group (n = 6). All procedures involving animals were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at Osaka Medical College.

2.3. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in plasma

Plasma was separated from the blood samples by centrifugation at 3000 rpm for 15 min at 4 °C. Measurements of plasma activities of AST and ALT were performed by SRL Co. Ltd. (Tokyo, Japan).

2.4. HMGB1 in plasma

HMGB1 in plasma was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Shino-test Co., Sagamihara, Japan) according to the manufacturer's instructions.

2.5. Active NF-κB p65 in liver

To measure NF-κB p65, nuclear proteins were isolated from fresh mouse liver using an NF-κB p65 ELISA kit (NOVUS Biologicals, Abingdon, UK) according to the manufacturer's instructions. Then, NF-κB p65 was measured using the NF-κB p65 ELISA kit.

Protein concentrations of the nuclear fraction were assayed using the bicinchoninic acid Protein Assay Reagent (Pierce, Rockford, IL) with bovine serum albumin as the standard.

2.6. Real-time polymerase chain reaction (RT-PCR)

Liver total RNA was extracted using the Trizol reagent (Life Technologies, Rockville, MD) and subsequently dissolved in RNase-free water (Takara Bio Inc. Otsu, Japan). Total RNA (1 μg) was transcribed into cDNA with Superscript VIRO (Invitrogen, Carlsbad, CA). Levels of mRNA were measured by RT-PCR on a LightCycler with software (Roche Diagnostics, Tokyo, Japan) using TaqMan fluorogenic probes. Primers and probes for RT-PCR of TNF-α, myeloperoxidase (MPO), and 18S ribosomal RNA (rRNA) were designed by Roche Diagnostics. The primers were as follows: 5′-aggcgaagattactgccaag-3′ (forward) and 5′-catggctatgaggtagagacagg-3′ (reverse) for TNF-α, 5′-ctgaatcctcgatggaatgg-3′ (forward) and 5′-ccatggcccctacaatctt-3′ (reverse) for MPO, and 5′-gcaattattccccatgaacg-3′ (forward) and 5′-gggacttaatcaacgcaagc-3′ (reverse) for 18S rRNA. The probes were as follows: 5′-agccccag-3′ for TNF-α, 5′-ccaggagg-3′ for MPO, and 5′-ttcccagt-3′ for 18S rRNA. mRNA levels of TNF-α and MPO were normalized to that of 18S rRNA.

2.7. Histological analysis

The liver tissue specimens were fixed with Carnoy's fixative in 10% methanol overnight. The fixed liver tissues were embedded in paraffin, and then cut at a thickness of 5 μm. The sections were mounted on silanized slides (Matsunami, Kishiwada, Japan) and deparaffinized with xylene and ethanol. The severity of hepatic histological changes was assessed using hematoxylin and eosin (HE) staining.

The procedure for immunohistochemical analysis of MPO has been previously described (17). Sections were incubated with anti-MPO antibody (Abcam Inc. Cambridge, MA) followed by a reaction with appropriate reagents from a streptavidin-biotin peroxidase kit (Dako LSABkit; Dako Co., Carpinteria, CA) and 3-amino-9-ethylcarbazole, which was used for color development. The sections were lightly counterstained with hematoxylin.

2.8. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Significant differences among mean values of multiple groups were evaluated using a one-way analysis of variance followed by Fisher's test. The cumulative survival in each group was determined using the Kaplan–Meier method, and survival was compared between groups with a log-rank test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of TM-α on survival rate after LPS/GalN administration

Twenty-four hours after LPS/GalN administration, the survival rates were 15% and 55% in the placebo- and TM-α-treated groups, respectively (Fig. 1). However, additional mortality was not observed in either group after 24 h (Fig. 1). The survival rates