Metallothionein-Null Mice Are Sensitive to Endotoxine/D-Galactosamine-Induced Hepatotoxicity

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(Received February 19, 2001; Accepted March 14, 2001)

Metallothionein (MT), which is a low-molecular weight, cysteine-rich, metal-binding protein, is induced during acute-phase reactions. However, the specific function of MT in the acute-phase response remains to be elucidated. We previously reported that MT-I, II deficient (MT-null) mice are highly sensitive to the lethal effects of lipopolysaccharide (LPS)/D-galactosamine (GalN). We designed the present study to clarify the major cause of the differences in the sensitivity to the lethal effects of LPS/GalN between wild type and MT-null mice. We found that histological grade of hepatocellular necrosis, induced by LPS/GalN, was greater in MT-null mice than in wild type mice. Therefore, the present findings suggest that MT induction has the potential as an attenuator of LPS/GalN-induced liver necrosis.

Key words —— metallothionein, liver necrosis, lipopolysaccharide, D-galactosamine

INTRODUCTION

Bacterial lipopolysaccharide (LPS) is the primary pathogenic factor in gram-negative bacteria. Mice injected with a high dose of LPS suffer multiple organ failure, as characterized by circulatory response syndrome and endotoxic shock. Following administration of LPS, the liver responds by markedly increasing the synthesis of a subset of serum proteins, known as the acute-phase proteins.[1] Some of these acute-phase proteins have been reported to protect experimental animals from lethal endotoxemia or other inflammatory challenges.[2–7] The induction of a number of acute-phase proteins is mediated by interleukin (IL)-6.[8,9] Metallothionein (MT), which is a low-molecular weight, cysteine-rich, metal-binding protein, is known to be induced during the acute-phase response.[10] MT induction during the acute-phase response is also mediated by IL-6.[11] However, the specific function of MT during the acute-phase response remains to be elucidated. We previously reported that MT-I, II deficient (MT-null) mice are highly sensitive to the lethal effects of LPS/D-galactosamine (GalN).[12] However, the major cause of the differences in sensitivity to the lethal effects of LPS/GalN between wild type and MT-null mice was unknown. We designed the present study to clarify the major cause of the differences in the sensitivity to the lethal effects of LPS/GalN between wild type and MT-null mice. Here, we found that histological grade of hepatocellular necrosis, induced by LPS/GalN, was greater in MT-null mice than in wild type mice. Therefore, the present findings suggest that MT induction has a potential as an attenuator of endotoxic shock.

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MATERIALS AND METHODS

Materials —— LPS from *Escherichia coli* O26 : B5 was obtained from Difco laboratories (Detroit, MI). Recombinant mouse tumor necrosis factor (TNF)-α was obtained from Pepro Tech, Inc. (Canton, MA, U.S.A.). GalN was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Animals —— Male 8 to 12 week-old MT-null mice and their corresponding controls (129/Sv) were pro-

vided by Jackson Laboratory (Bar Harbor, ME) and maintained as a closed colony in our laboratory. All mice were housed under conditions of controlled temperature (23–24°C) and light (12 hr light and 12 hr dark). Food and tap water were provided *ad libitum*.

Animal Experiments —— The experiments were conducted in accordance with the institutional guidelines of Osaka University. Wild type and MT-null mice were intraperitoneally administrated with LPS (100 μg/kg) and GalN (700 mg/kg). The survival rate of mice over the next 24 hr was recorded.

Measurement of Prothrombin Time —— For plasma preparation, blood (450 μl) was collected via a syringe containing 50 μl of 0.1 M sodium citrate. Plasma was then obtained by centrifugation for 5 min at 10000 g. Prothrombin time (PT) was measured using Thrombo check PT® according to the manufacturer's protocol (Kokusai Shiyaku, Kobe, Japan).

Histopathology —— At 6 hr after the injection of LPS/GalN, the whole bodies of wild type and MT-null mice were preperfused with heparin-containing phosphate buffered saline under ether anesthesia, and then fixed by perfusion with 10% neutral buffered-formalin for 10 min. The brain, liver, kidney, spleen, heart and lungs from all mice were collected and embedded in paraffin. These samples were sectioned, and stained with hematoxylin and eosin for histopathological examination.

Statistics —— Significant differences in survival rate were analyzed by the Kaplan-Meier (Product-Limit) method and log rank test. Significant differences in the other findings were analyzed by analysis of variance (ANOVA) and Fisher's protected least-significant difference (PLSD) test. Differences between groups were considered to be significant at $p < 0.05$ level.

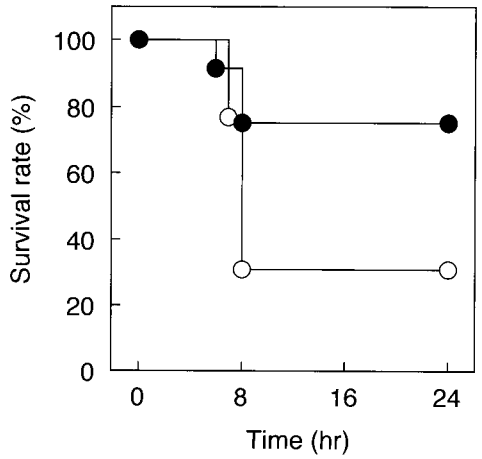

Fig. 1. Survival Curve of Wild Type and MT-Null Mice in Response to Intraperitoneal Administration of LPS/GalN

Significant differences were analyzed by the Kaplan-Meier (Product-Limit) method and log rank test. Survival rates in wild type (●, $n = 12$) and MT-null mice (○, $n = 13$) were significantly different at $p < 0.05$.

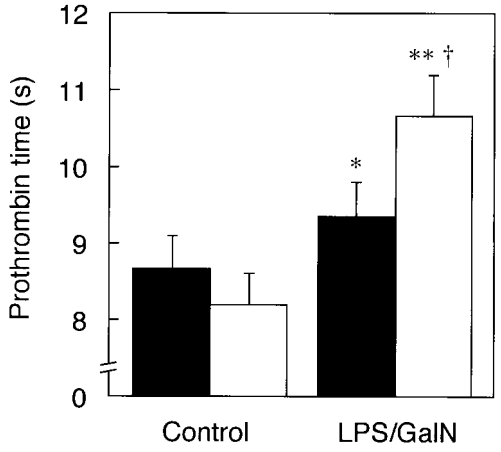

Fig. 2. Prothrombin Time after Administration of LPS/GalN in Wild Type and MT-Null Mice

Prothrombin time in wild type (■) and MT-null (□) mice before and 5 hr after administration of LPS/GalN. Values represent the means ± S.D. ($n = 3$–4). Significantly different from the control group ($^{*}p < 0.05$; $^{**}p < 0.001$), and significaintly different from wild type mice ($^{\dagger}p < 0.01$).

RESULTS AND DISCUSSION

We previously reported that MT-null mice are highly sensitive to the lethal effects of LPS/GalN.[12] However, the major cause of the differences in the sensitivity to the lethal effects of LPS/GalN between wild type and MT-null mice remained unknown. Here, we found that historical grade of hepatocellular necrosis was greater in MT-null mice than in wild type mice. This was the major cause of the differences in the sensitivity to the lethal effects of LPS/GalN between wild type and MT-null mice.

As shown in Fig. 1, MT-null mice exhibited high sensitivity to the lethal effects of LPS/GalN compared to wild type mice. At 24 hr after LPS/GalN administration, the survival rate was only 30.7% for MT-null mice compared with 75.0% for wild type mice ($p < 0.05$). After administration of LPS/GalN, a number of clinical paramaters changed: an increase of the levels of liver transaminase in the circulation and the plasma clotting time, and a decrease in body temperature.[6] Five hours after administration of LPS/GalN, an enhanced PT was found in wild type

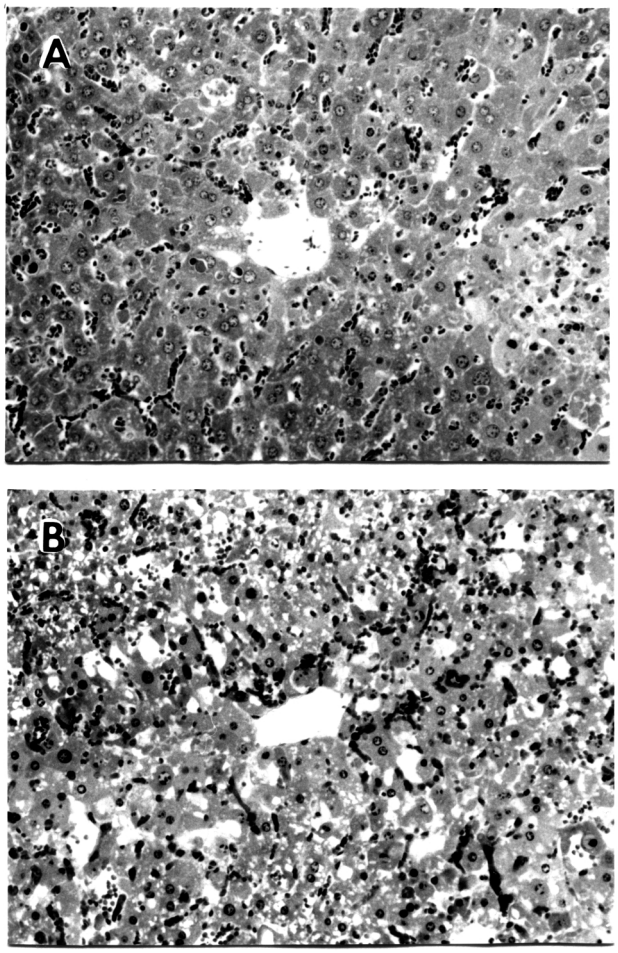

Fig. 3. Light Micrographs of the Liver Sections 6 hr after Administration of LPS/GalN

A) Slight focal hepatocellular necrosis with inflammatory cell infiltration in the centrilobular region of the liver of a wild type mouse. B) Severe focal hepatocellular necrosis with inflammatory cell infiltration in the centrilobular region of the liver of an MT-null mouse. Magnification × 175.

and MT-null mice (Fig. 2). The increase in PT was greater in MT-null mice than in wild type mice ($p < 0.01$). The enhanced PT was caused by hepatocellular necrosis or disseminated intravascular coagulation (DIC). Histopathological examination was performed to clarify the major cause of the differences in the sensitivity to the lethal effects of LPS/GalN between wild type and MT-null mice. Hepatocellular necrosis, hepatocellular swelling and/or hepatocellular vacuolation were histopathologically observed in wild type and MT-null mice (Fig. 3). Hepatocellular necrosis in MT-null mice was moderate to severe. In addition, the grade of the lesion was much greater in MT-null mice than that in wild type mice. Therefore, these findings indicated that the major cause of the differences in the sensitivity to the lethal effects of LPS/GalN between wild type and MT-null mice was the differences in the sensitivity to the effects of LPS/GalN-induced hepatotoxicity. At the same time, perivascular and/or diffuse cell infiltrations in the lung and atrophy of red pulps in the spleen were observed in wild type and MT-null mice (data not shown). These lesions might be caused by LPS/GalN, however, these were slight changes, and were not the major cause of the differences in the sensitivity to the lethal effects of LPS/GalN between wild type and MT-null mice. However, DIC was not induced in the brain, liver, kidney, spleen, heart and lungs of wild type or MT-null mice under our experimental conditions. In addition, no other marked changes were observed in the brain, kidney and heart in either mouse group.

Mice develop drastic liver failure after administration of LPS/GalN.[13] LPS/GalN-induced liver necrosis was mainly mediated by TNF-α.[14] TNF-α leads the GalN-sensitized hepatocytes to apoptotic cell death. Subsequently, polymorphonuclear leukocytes (PMNs) accumulate in sinusoids, and the transendothelial migration and adherence of PMNs to hepatocytes are observed.[15] PMNs release reactive oxygen species (ROS) and mediate hepatocellular injury.[16] It was suggested that MT can act as an attenuator of apoptosis[17] and inhibitor of ROS-induced cytotoxicity.[18] These actions of MT might be involved in the prevention of LPS/GalN-induced hepatotoxicity. However, we previously reported that the mRNA level of α_1-acid glycoprotein (AGP) in the response to LPS/GalN was decreased in MT-null mice compared with wild type mice.[12] In addition, MT may have the potential to prevent LPS/GalN-induced lethality, at least through the attenuation of AGP induction. The present findings were concomitant with our previous findings, since AGP is able to prevent LPS/GalN-induced hepatotoxicity.

Liver necrosis is recognized as a life-threatening complication in endotoxic shock. The endotoxin-induced liver necrosis is mainly mediated by TNF-α. Liver necrosis induced by TNF-α has proven to be one of the dose–limiting toxicities in clinical trials with TNF-α. Induction of liver necrosis by alcohol[19] or by hepatitis B virus[20] was found to correlate with increased TNF-α levels. Therefore, prevention of liver necrosis is needed to increase the therapeutic value of TNF-α and to efficiently cure othere diseases with liver necrosis complications. It is suggested that MT acts as a protectant against LPS/GalN-induced liver necrosis. The present findings suggest that MT induction has potential for clinical applications in chemotherapy. Experiments that in-

vestigate the precise action of MT on inhibition against liver necrosis will be of great interest.

Acknowledgements This work was supported in part by a Grant in Aid for Scientific Research (No. 12877372) from the Ministry of Education, Science, Sports and Culture, and also supported by a grant from Takeda Science Foundation.

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