A Protective Role for Metallothionein in Acetaminophen-caused Liver Toxicity *via* Oxidative Stress

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To investigate the possible involvement of metallothionein (MT) in acetaminophen (AC)-induced liver damage, MT-I and MT-II gene knock-out transgenic mice (MT-null mice) and wild-type control mice were i.p. treated with AC at a single dose of 250 mg/kg and compared. At 24 h after AC treatment severe liver damage characterized by necrosis of hepatocytes and increased serum GPT activity was found in MT-null mice while a limited degree of change such as a slight increase in serum GPT activity without necrosis was observed in wild-type mouse liver. MT-null hepatocytes also showed elevated PCNA (proliferating cell nuclear antigen)-positive activity around the necrosis area. AC administration resulted in an increase in lipid peroxidation in MT-null mice, but not in the wild-type mice. The liver in the wild-type mice showed an increased MT amount after AC treatment. These results indicate that MT acts as an endogenous defensive factor against AC-induced hepatotoxicity *via* oxidative stress.

Key words —— acetaminophen, lipid peroxidation, liver damage, metallothionein

INTRODUCTION

An excessive dose of acetaminophen (AC), a widely used analgesic and antipyretic agent, causes liver damage in human and experimental animals.[1] AC-induced primary hepatotoxicity is thought to be exerted by a free radical-mediated pathway mainly originating from *N*-acetal-*p*-benzoquinone imine (NAPQI), the major reactive metabolite.[2] Generally, NAPQI is detoxified by conjugation with glutathione (GSH), and under depletion of GSH, NAPQI will bind to other important cellular macromolecules, which results in liver damage.[2] The involvement of oxidative stress has also been found in the process of the AC-induced free radical-mediated pathway, since the hepatic lipid peroxidation (LPO) level was elevated after AC treatment both *in vivo* and *in vitro*.[3,4] The free radical hypothesis was supported by the fact that its hepatotoxicity has been suppressed by several free radical scavengers and antioxidant supplementation,[5,6] suggesting that endogenous anti-free radical/antioxidative capacity of the tissue is important for suppressing AC hepatotoxicity.

Recently, metallothionein (MT)-null transgenic mice that are deficient in MT-I and II, the major isoforms of MT, were produced by the gene targeting technique.[7] The mice and their cells were very susceptible to the toxicity of paraquat and *tert*-butylhydroperoxide,[8,9] and proved to be a good model for studying its normal function and the consequences of its deficiency. In order to evaluate possible protective roles of MT against toxicity of various chemicals through a free radical pathway, we studied the sensitivity of MT-null mice to AC-induced hepatotoxicity.

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MATERIALS AND METHODS

Chemicals and Reagents —— LPO measurement kit (Lipoperoxide-Test Wako), AC and 10% neutral buffered formalin solution were purchased from Wako Pure Chemical Industries (Osaka, Japan). Monoclonal mouse antibody against proliferating cell nuclear antigen (anti-PCNA, Clone PC10) was obtained from Dako Japan Co.(Tokyo). Second antibody (biotinylat-

ed goat anti-mouse IgG) and ABC reagents were purchased from Vector Laboratories (Burlingame, CA, U.S.A.). GSH measurement kit (BIOXYTECH GSH-400) was obtained from Oxis International (Portland, OR, U.S.A.).

Transgenic Mice and Treatment —— MT-null mice and wild-type mice having a genetic background of C57BL6/OLA129 were kindly provided by Dr. A. Choo.[7] The mice were housed in screen-bottomed stainless steel cages which were kept in a ventilation cabinet. The animal facility was maintained under conditions of a 12-h light/dark cycle, temperature of 24±2℃, relative humidity of 55±10% and negative atmospheric pressure. The mice received mouse chow and filtered tap water *ad libitum*. Animals received humane care throughout the experiment according to the guidelines for animal welfare at the National Institute for Environmental Studies.

For the experiment, 10-week-old female mice were used. Each group consisted of 5 mice. AC was administered i.p. in a single dose of 250 mg/kg, dissolved in warm saline. Control mice received the same volume (10 ml/kg body weight) of warm saline. At 24 h after AC treatment, the mice were sacrificed by an overdose of ether. The serum was collected for analyses of glutamate pyruvate transaminase (GPT), glutamate oxaloacetic transminase (GOT) and lactate dehydrogenase (LDH). A portion of liver tissues was collected for histological observations and the rest was kept at −80℃ until biochemical analyses.

Biochemical Analyses —— Serum GPT, GOT and LDH activities were determined by using an automatic dry-chemical analyzer (Spotchem SP-4410, Kyoto Daiichikagaku, Kyoto). Malondialdehyde (MDA) and GSH in the liver were determined by Wako LPO kit and BIOXYTECH GSH-400 kit, respectively. MT determination in tissues was performed by an RIA method described previously.[10]

Histological Examination and Immunochemical Analysis —— Liver specimens were fixed in 10% neutral-buffered formalin solution and processed for paraffin embedding. Tissue sections (5 μm) were placed either on a glass slide for hematoxylin and eosin staining or on a poly-L-lysine coated glass slide for PCNA histochemical staining. For PCNA staining, deparaffinized tissue sections were treated with 3% hydrogen peroxide solution for 5 min. Following preincubation with 1% normal goat serum in phosphate-buffered saline for 30 min, PC10 (dilution 1 : 300) was applied to each section for 2 h at room temperature. Biotinylated goat anti-mouse IgG serum was used as secondary antibody (dilution 1 : 300), and the immunoreaction was visualized by the ABC method using diaminobenzidine as substrate. Mayer's hematoxylin was used for counterstaining. The control section was treated with normal mouse serum instead of PC10.

Statistical Analysis —— The data were analyzed by Student's *t*-test for comparison of means between MT-null mice and wild-type mice. The statistical significance was set below 0.05.

RESULTS AND DISCUSSION

Changes in hepatic MT and GSH concentrations are shown in Fig. 1. The MT concentration in the liver of untreated wild-type mice was 14 μg/g tissue and increased about 4 times by treatment with AC. In the MT-null mice, the hepatic MT was not detected (<0.2 μg/ g tissue), confirming the deficiency of MT protein. No significant change in GSH concentrations was observed in the liver of both strains of mice at 24 h after AC treatment.

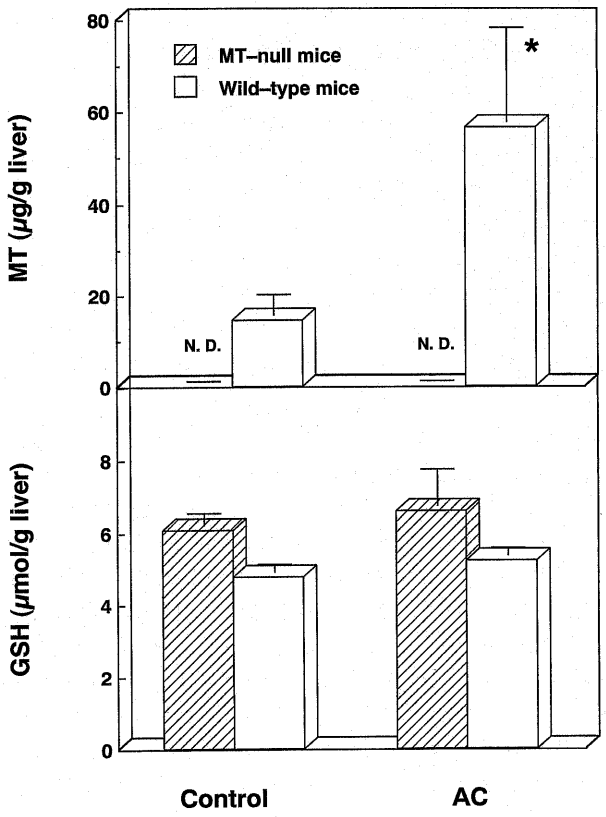

Fig. 1. MT and GSH Levels in the Liver of MT-null Mice and Wild-type Mice at 24 h after AC Injection (250 mg/kg, i.p.)

Values are means ± S.E. of 5 mice. N.D., not detected.* Significantly different from control wild-type mice ($p<0.05$).

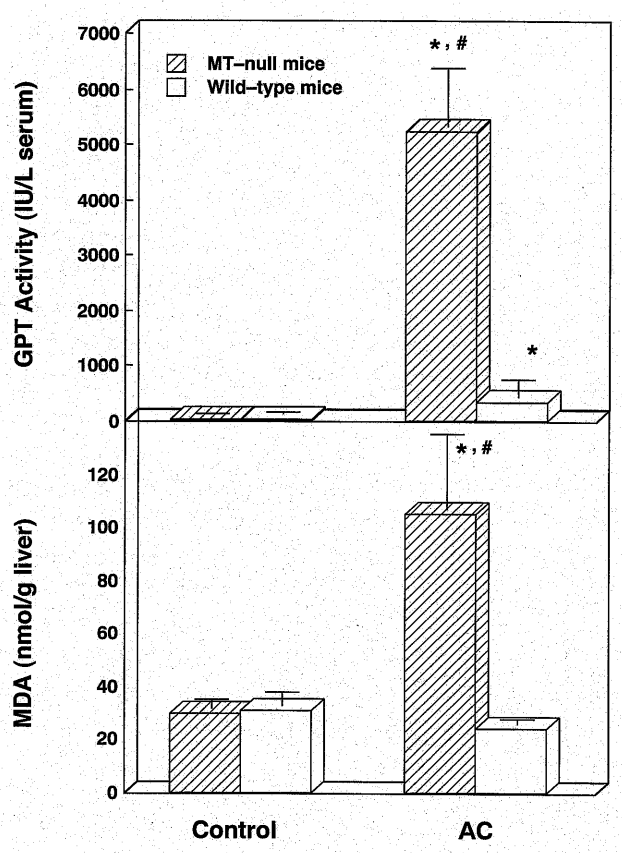

Fig. 2. GPT Activity in the Serum and MDA Levels in the Liver of MT-null Mice and Wild-type mice at 24 h after AC Injection (250 mg/kg, i.p.)

Values are means ± S.E. of 5 mice. *Significantly different from corresponding control group ($p<0.05$). #Significantly different from AC-treated wild-type mice ($p<0.05$).

Changes in serum GPT activity and lipid peroxidation in the liver by AC treatment are shown in Fig. 2. Serum GPT activity was slightly increased in wild-type mice whereas it was considerably elevated in response to AC in MT-null mice: approximately 15 times higher than wild-type mice treated with same dose of AC. Serum GOT and LDH activities showed a similar tendency to GPT activity (data not shown).

At 24 h after AC treatment MDA level (as an indicator of LPO) in the liver of MT-null mice was increased approximately 4 times higher than that of untreated control mice, while there was no change in wild-type mice by AC treatment (Fig. 2). In the course of the present study, Rofe and coworkers[11] have reported that MT-null mice were more susceptible to AC-induced hepatotoxicity than wild-type mice, as characterized by increased serum GPT and LDH activity but that no determination was described for MDA. Utilizing MT-null mice, we demonstrated that the degree of LPO was more pronounced under MT deficient conditions. The elevated level of MT in wild-type mice after AC treatment is thought to be a response after AC-induced oxidative stress. The MT-null mice are also susceptible to the toxicity of paraquat and *tert*-butylhydroperoxide.[8,9] The antioxidant effect of MT, which is a potent hydroxyl radical scavenger *in vitro* and can also inhibit hydroxyl radical-induced DNA damage, was higher than that of GSH *in vitro*.[12]

The liver damage found in AC-treated MT-null mice was histologically identified as centrilobular necrosis (Fig. 3B), but administration of AC did not cause necrotic changes in wild-type liver (Fig. 3D) although serum GPT activity was slightly elevated. Both apoptosis and necrosis were observed in CD-1 mice by AC administration.[13] Oxidative stress can cause cell damage and is suggested as a mediator of apoptosis.[14] These results suggest that MT may contribute to preventing AC-induced cell death through suppressing oxidative stress. When zinc was administrated to CD-1 mice to enhance the biosynthesis of MT before AC administration, it was found that zinc could prevent AC-induced hepatotoxicity,[15] suggesting a protective role of MT against AC toxicity. Using MT-null mice, the present study could negate other effects of zinc ions beside MT induction and indicated that basal amounts of MT in the liver play an important role in preventing against AC-induced hepatotoxicity.

These liver tissues showed many PCNA-positive nuclei around the necrotic area (Fig. 3F). PCNA-positive nuclei were occasionally observed in hepatocytes of the wild-type mice (Fig. 3H) while no PCNA-immunostaining was observed with preimmune mouse serum when both types of liver were treated with or without AC (data not shown). In the necrotic area, the cytoplasm of degenerated cells was stained brown (Fig. 3F), showing the elevated level of IgG due to AC-induced inflammatory response. Without intentionally using control goat serum to mask non-specific staining, it was possible to demonstrate that not only PCNA but also mouse IgG can react with the second antibody (an anti-mouse IgG) used for PCNA staining. The PCNA has been suggested as a useful marker for rodent hepatocyte proliferation.[16] The enhanced proliferation of hepatocytes in the MT-null mice in the present study, indicated an acute proliferative

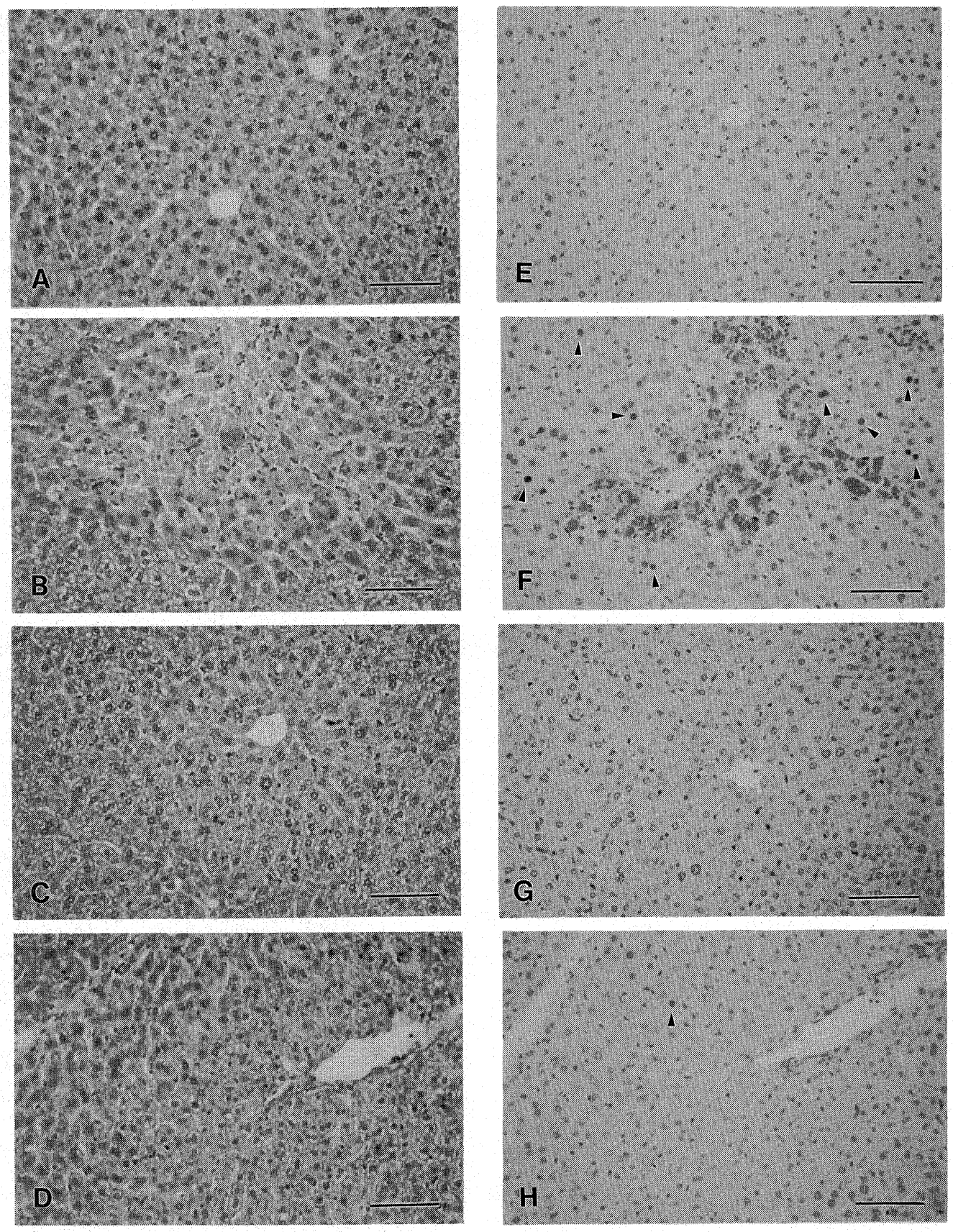

Fig. 3. Histopathological Changes in the Liver of MT-null Mice and Wild-type Mice Treated with AC (250 mg/kg i.p.)

A, B, C, D, Hematoxylin and eosin staining. E, F, G, H, Immunohistochemical detection of PCNA. PCNA-positive nuclei were stained dark-brown (arrowhead) in the nucleus of hepatocyte. The degenerated and necrotic cytoplasm was stained brown. A, E, control MT-null mouse; B, F, AC-treated MT-null mouse; C, G, control wild-type mouse; D, H, AC-treated wild-type mouse. Bar, 100 μm.

response to AC-induced acute injury, which was consistent with the finding of elevated thymidine incorporation in the AC-treated rat liver cells.[17]

It is thought that NAPQI is responsible for attacking macromolecules *via* free-radical inducing pathway[2] and that GSH plays a major role in scavenging their free radicals. Nevertheless, it is reasonable to suggest that MT, as a source of sulfhydryls, may scavenge electrophilic intermediates of AC and/or free radicals related to AC metabolism. In this way, other important cellular molecules are protected from AC attack by this additional protective mechanism.

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