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Gadolinium Chloride Pretreatment Prevents Cadmium Chloride-Induced Liver Damage in Both Wild-Type and MT-Null Mice[1]

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The heavy metal cadmium (Cd) causes hepatotoxicity upon acute administration. Kupffer cells, the resident macrophages of the liver, have been suggested to play a role in Cd-induced hepatotoxicity. Gadolinium chloride ($GdCl_3$) may prevent Cd-induced hepatotoxicity by suppressing Kupffer cells. However, $GdCl_3$ also induces the Cd-binding protein, metallothionein (MT). Therefore, this study was conducted to determine whether $GdCl_3$ prevents Cd-induced hepatotoxicity via the induction of MT. Hepatic MT and Kupffer cell counts were analyzed 24 h after wild-type (WT) mice were administered saline or 10, 30, or 60 mg $GdCl_3$/kg. $GdCl_3$ induced MT in a dose-dependent manner without affecting nonprotein sulfhydryl content. All examined doses of $GdCl_3$ were effective at eliminating Kupffer cells from the liver. To examine the hepatoprotective effects of $GdCl_3$, WT and MT-null mice were pretreated with saline or 10, 30, or 60 mg $GdCl_3$ 24 h prior to a hepatotoxic dose of Cd (2.5 mg Cd/kg). Blood and livers were removed 16 h later and analyzed for hepatotoxicity as well as MT, Cd, and Kupffer cell content. Hepatotoxicity was alleviated in both WT and MT-null mice that were pretreated with 30 or 60 mg $GdCl_3$/kg, indicating that MT induction is not required for the hepatoprotective effects of $GdCl_3$. Hepatic Cd content was not decreased by $GdCl_3$, demonstrating that $GdCl_3$ does not negatively affect Cd distribution to the liver. Kupffer cells were depleted at all three doses of $GdCl_3$, whereas hepatoprotection was only observed at doses of 30 and 60 mg $GdCl_3$/kg. This does not rule out Kupffer cells in the mechanism of Cd-induced hepatotoxicity, but it does suggest that $GdCl_3$ exerts hepatoprotective effects on the liver aside from depleting Kupffer cells. In summary, these data substantially rule out MT induction and decrease the importance of Kupffer cells as mechanisms of $GdCl_3$-induced protection from Cd-induced hepatotoxicity. © 2002 Elsevier Science (USA)

Key Words: cadmium; liver; hepatotoxicity; necrosis; gadolinium chloride; metallothionein; MT-null.

The heavy metal cadmium (Cd) is an industrial and environmental pollutant. It is toxic to several tissues, most notably causing hepatotoxicity upon acute administration and nephrotoxicity upon chronic exposure. Histological evaluation of liver injury reveals that acute toxicity is comprised of hepatocellular swelling, sinusoidal congestion, pyknosis, and karyorrhexis (Dudley *et al.*, 1982). In a time-course study on Cd-induced hepatotoxicity, early cellular changes occur in the rough endoplasmic reticulum and nucleus (Dudley *et al.*, 1984). Later alterations include swelling of mitochondria and endoplasmic reticulum, loss of ribosomes, and appearance of fibrillar material within the cytoplasm. These cellular changes may result in both apoptosis and necrosis (Habeebu *et al.*, 1998).

Kupffer cells, the resident macrophages of the liver, have been suggested to play a role in Cd-induced hepatotoxicity. Kupffer cell activation by Cd was first noted by the identification of cytoplasmic vacuolization (Hoffmann *et al.*, 1975) and increased colloidal carbon clearance, which is indicative of increased phagocytic activity (Sauer *et al.*, 1997; Yamano *et al.*, 2000). Elimination of Kupffer cells with gadolinium chloride ($GdCl_3$) alleviated Cd-induced hepatotoxicity in rats (Sauer *et al.*, 1997; Yamano *et al.*, 2000) but failed to protect cultured hepatocytes (Badger *et al.*, 1997). Several reports have examined increases in cytokine expression in response to Cd administration. Tumor necrosis factor-α (TNF-α) is weakly induced by Cd both *in vitro* (Szuster-Ciesielska *et al.*, 2000; Marth *et al.*, 2000) and *in vivo* (Kayama *et al.*, 1995b). Pretreatment with antibodies against TNF-α abrogated Cd-induced expression of acute phase proteins but did not decrease Cd-induced hepatotoxicity (Kayama *et al.*, 1995b). In contrast, Yamano *et al.* (2000) determined that TNF-α was not increased within 24 h of Cd administration. Other cytokines, including interleukin-1α (IL-1α), interleukin-1β (IL-1β), interferon-γ (IFN-γ), and interleukin-6 (IL-6) (Kayama *et al.*, 1995a) are also increased after Cd treatment, although their relevance is less well defined (Kayama *et al.*, 1995b; Liu *et al.*, 1999; Yamano *et al.*, 2000; Marth *et al.*, 2000). Taken together, these data strongly suggest a role for Kupffer cells in Cd-induced hepatotoxicity.

Glutathione (GSH), the primary cellular nonprotein thiol, plays a role in the detoxication of Cd, although the exact role GSH plays in Cd-induced hepatotoxicity is not accurately defined. Depletion of GSH with phorone, diethyl maleate, or

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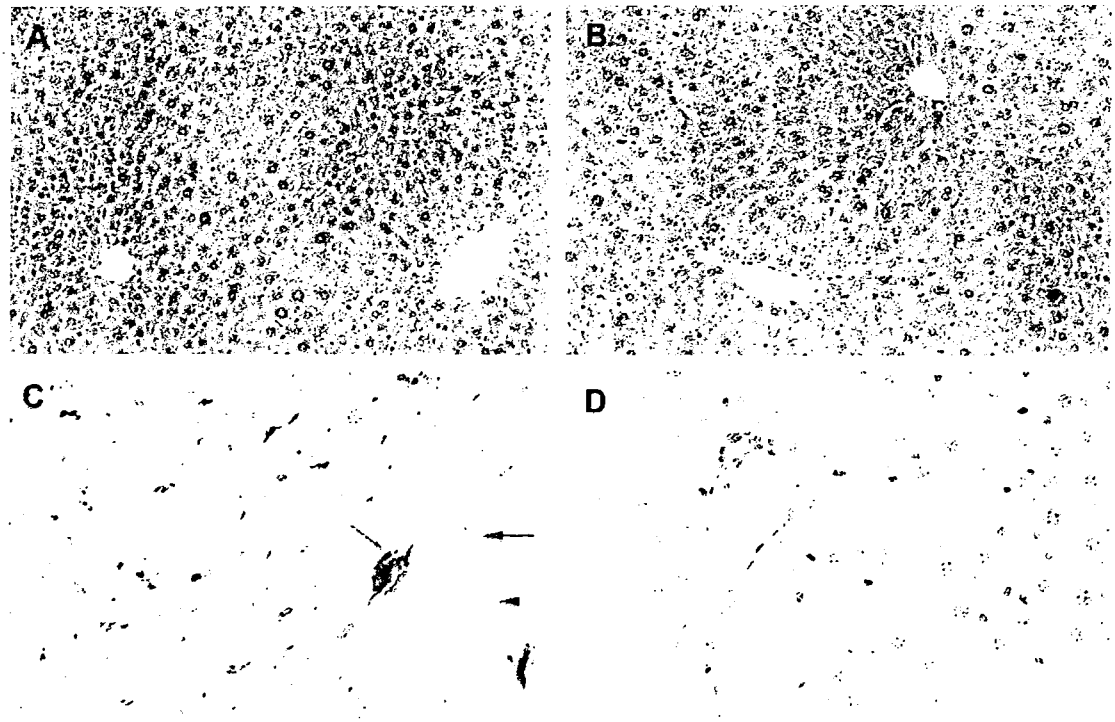

FIG. 1. Photomicrographs of wild-type mouse livers 24 h after $GdCl_3$ administration. Liver sections were prepared as described under Materials and Methods and visualized with either routine H & E staining (A and B, 200×) or immunohistochemical visualization of Kupffer cells (C and D, 400×). Photomicrographs are representative of mouse liver 24 h after administration of saline (A and C) or 60 mg $GdCl_3$ (B and D). Brown-staining cells were positive for the F4/80 antigen and were counted as Kupffer cells (C, inset, 1000×, thin arrow). Other cell types represented are endothelial cells (C, inset, arrowhead) and parenchymal cells (C, inset, thick arrow).

buthionine sulfoximine increases sensitivity to both Cd-induced lethality and hepatotoxicity (Dudley and Klaassen, 1984; Singhal *et al.*, 1987). Glutathione is also critical for the biliary elimination of Cd from the liver (Dijkstra *et al.*, 1996; Sugawara *et al.*, 1996). However, the protective effect may also be due to the reduction of Cd-induced oxidative stress by GSH (Shaikh *et al.*, 1999). Therefore, GSH plays an important and perhaps multifunctional role in the protection against Cd-induced liver injury.

Metallothioneins (MT) are small, cysteine-rich proteins that bind Cd with high affinity (Klaassen *et al.*, 1999). Pretreatment with low doses of zinc or Cd provides protection against subsequent hepatotoxic doses of Cd (Goering and Klaassen, 1984a,b). This protection appears to be due to induction of MT and subsequent redistribution of Cd to the cytoplasm, where it is sequestered by MT and thus detoxified (Goering and Klaassen, 1983). MT-null mice are not protected from subsequent administration of Cd, thus confirming the requirement of MT in the mechanism of this protective effect (Liu *et al.*, 1996). $GdCl_3$ has also been shown to induce MT, although the significance of this induction is unknown (Sauer *et al.*, 1997).

These observations demonstrate that $GdCl_3$ exerts a variety of effects on the cells within the liver, including depletion of Kupffer cells as well as induction of MT. It is unknown which of these effects protects against Cd-induced hepatotoxicity. Therefore, this study was designed to determine whether $GdCl_3$ prevents Cd-induced hepatotoxicity by depletion of Kupffer cells or induction of MT.

MATERIALS AND METHODS

Animals. Wild-type (WT) (129SvIm/J, Jackson Labs, Bar Harbor, ME) and mutant mice with disrupted MT-I and MT-II genes (MT-null) (Masters *et al.*, 1994) were used throughout the study. Mice were housed in an AAALAC-certified facility at 70 ± 2°F with a 12-h light/dark cycle and were fed laboratory mouse chow (Purina, St. Louis, MO) and water *ad libitum*.

Chemical treatment of mice. To determine the hepatic effects of $GdCl_3$, male WT mice (8 weeks old, approximately 25 g, $n = 5$) were administered saline or various doses of 10, 30, or 60 mg $GdCl_3$ into the tail vein in a volume

of 10 ml/kg or approximately 250 μl per mouse. Blood and liver were collected 24 h after dosing. Blood was processed for serum enzyme activity analyses. Livers were removed and a portion of the left lobe of each liver was fixed in 10% neutral buffered formalin. After 24 h in formalin, the fixed liver samples were switched to 70% ethanol for storage. The remainder of the liver was snap frozen in liquid nitrogen and stored at −80°C. To examine the hepatoprotective effects of $GdCl_3$, male and female WT and MT-null mice (8 weeks old, approximately 25 g, $n = 7–8$) were injected with saline or 10, 30, or 60 mg $GdCl_3$ iv 24 h prior to a hepatotoxic dose of $CdCl_2$ (2.5 mg Cd/kg iv). Sixteen hours after Cd administration, blood and liver were collected and processed as described for the first experiment.

Serum enzyme activity assays. Biochemical evaluation of liver injury was performed by quantifying serum activities of alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) spectrophotometrically, using Sigma test kits (Sigma Chemical Company, St. Louis, MO) according to the manufacturer's instructions.

Histopathology. Liver samples were taken consistently from the left lobe of the liver and processed by standard histological techniques. Briefly, samples were fixed in formalin for 24 h and then switched to ethanol for storage. Liver sections were then processed routinely and embedded in paraffin blocks. Slides were prepared (5 μm) and stained with hematoxylin and eosin. The slides were blinded and analyzed by light microscopy for liver injury. The grade of liver injury was analyzed semiquantitatively with six scores of severity per liver section: 0 = none; 1 = minimal (>2 foci of single cell necrosis); 2 = mild (at least 5 areas of focal necrosis); 3 = moderate (at least five foci of zonal necrosis); 4 = severe (lobular damage, with many viable lobules); 5 = global (severe lobular damage, few areas of viability).

Immunohistochemistry. Liver samples were fixed and embedded as described above. Liver sections were cut (5 μm) and mounted on Superfrost Plus slides (Fisher Scientific, Fair Lawn, NJ). Briefly, slides were incubated with proteinase K (20 μg/ml) for 15 min at room temperature for antigen retrieval. Endogenous peroxidase activity was quenched by treatment of the slides with 3% H_2O_2 for 10 min. Treating the slides for 30 min with 5% normal rabbit serum in PBS blocked nonspecific binding. Kupffer cells were then detected with a primary antibody to the F4/80 macrophage surface antigen (MCAP497, Serotec, Inc., Raleigh, NC). A biotinylated anti-rat secondary antibody (BA-4001, Vector Laboratories, Burlingame, CA) specifically identified the primary antibody. The liver sections were then incubated with avidin and biotinylated horseradish peroxidase (HRP) and identified using the HRP substrate diaminobenzidine and counterstained with hematoxylin. Brown-staining cells with appropriate nuclear morphology and sinusoidal location were counted as Kupffer cells. Kupffer cells were quantified as the average number of F4/80-positive cells per field (400× magnification) from at least 20 randomly selected fields per section.

Metallothionein assay. Liver samples were prepared by homogenizing 1:3 (w/v) in 10 mM Tris–HCl buffer (pH 7.4), centrifuged at 13,000*g*, and the supernatant fractions were retained. Protein concentration in supernatants was determined by the bicinchoninic acid method using a BCA kit (Pierce, Rockford, IL). Supernatants were assayed and hepatic MT content was determined using the Cd–hemoglobin method as previously described (Eaton and Toal, 1982).

Hepatic nonprotein sulfhydryl (NPSH) assay. Hepatic NPSH was quantified using a method described previously (Ellman, 1959). Briefly, 100-mg liver samples were homogenized using a Kinematica Polytron homogenizer (Littau, Switzerland) in 5% TCA/EDTA and separated by centrifugation at 13,000*g*, and the supernatants were retained for NPSH analysis. Each reaction contained 176 μl of 0.1 M PO_4^{3-} buffer (pH 8), 16 μl of supernatant, and 8 μl of 5 mM 5,5′-dithio-bis(2-nitrobenzoic acid). Absorbance was quantified using a Biotek microtiter plate spectrophotometer (Winooski, VT) at 405 nm (analytical wavelength) and 690 nm (reference wavelength) and compared with a standard curve of known GSH concentrations.

Hepatic Cd assay. Liver samples (~1.0 g) were digested 1:3 (w/v) in concentrated HNO_3 at 100°C for 60 min. The digested samples were assayed for Cd content using a Perkin–Elmer atomic absorption spectrophotometer (Norwalk, CT) with an analytical wavelength of 228.8 nm. Sample Cd content was determined by comparing absorbance values to a standard curve of Cd solutions.

Statistics. One-way ANOVA was used to analyze data from the $GdCl_3$ dose–response study. Two-way ANOVA followed with a Duncan's multiple range test was used for all data from Cd-treated groups. For all experiments, the acceptable level of significance was chosen to be $p < 0.05$.

RESULTS

Assessment of hepatic effects of $GdCl_3$. Doses of 10, 30, and 60 mg $GdCl_3$/kg caused no overt toxicity in WT mice. Serum markers of hepatotoxicity, ALT and SDH were not increased or decreased after administration of any dose of $GdCl_3$ studied (data not shown). In addition to serum markers of hepatotoxicity, livers were examined for histological evidence of liver injury. After saline treatment, liver was histologically normal except for some mild hydropic change (Fig. 1A, 200×). After 60 mg $GdCl_3$, liver morphology was histologically indistinguishable from saline treatment (Fig. 1B, 200×). Kupffer cells were identified immunohistochemically and counterstained with hematoxylin (Figs. 1C and 1D). Nonparenchymal cells that were positive for the F4/80 antigen (brown-staining cells) and displayed appropriate nuclear morphology (inset, 1000×) were counted as Kupffer cells. Nuclear morphology of Kupffer cells can be contrasted with endothelial (inset, arrowhead) and parenchymal cell nuclei (inset, curved arrow). Kupffer cells were quantified as the number of F4/80-positive cells per high-power field (400×). Kupffer cells were abundant after saline administration (Fig. 1C, 400×). Similar analysis of liver sections after 60 mg/kg identified no F4/80-positive cells (Fig. 1D, 400×).

Quantitation of Kupffer cells after $GdCl_3$ administration. Kupffer cells were quantified as the number of F4/80-positive cells per field from at least 20 randomly selected fields per section. Kupffer cells were identified only in saline-treated control rats (Fig. 2A). At all doses of $GdCl_3$, no cells were positive for the F4/80 antigen. Nonparenchymal cells were also qualitatively analyzed by examining nuclear morphology to determine whether Kupffer cells were still present but not antigenic. However, no Kupffer cells could be positively identified morphologically.

Effect of $GdCl_3$ administration on hepatic MT and NPSH contents. Livers were processed and analyzed for metallothionein by the cadmium–hemoglobin radioassay (Eaton and Toal, 1982). Hepatic metallothionein increased in a dose-dependent manner after $GdCl_3$ administration (Fig. 2B). In saline-treated mice, MT was approximately 3 μg/g liver. In $GdCl_3$-treated mice, MT increased to 8, 18, and 42 μg/g liver at doses of 10, 30, and 60 mg $GdCl_3$/kg, respectively. These represent 3-, 6-, and 15-fold increases in hepatic MT concentration, respectively. In addition, liver was processed and analyzed for NPSH by the DTNB method (Ellman, 1959). NPSH

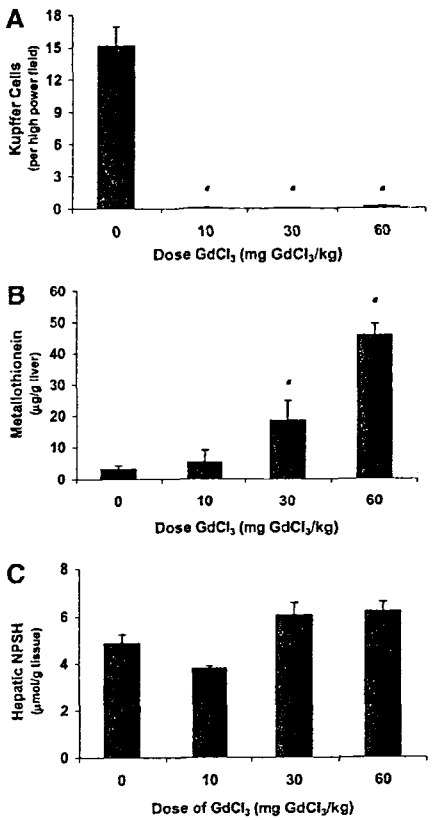

FIG. 2. Quantification of Kupffer cells, MT, and NPSH content 24 h after $GdCl_3$ administration. Wild-type mice (129Svlm/J) were administered saline or various doses of $GdCl_3$ (10, 30, or 60 mg $GdCl_3$/kg). Livers were collected 24 h after dosing. Livers were processed and analyzed for (A) Kupffer cells, (B) MT, and (C) NPSH content as described under Materials and Methods. (A) *Livers from $GdCl_3$-treated mice contained significantly fewer Kupffer cells than livers from saline-treated control mice ($p < 0.05$). (B) *Livers from $GdCl_3$-treated mice contained significantly greater MT than livers from saline-treated control mice ($p < 0.05$). Data are presented as means ± SE ($n = 5$).

concentrations were not significantly changed with any dose of $GdCl_3$ (Fig. 2C).

Histopathology of Cd-induced hepatotoxicity after saline or $GdCl_3$ pretreatment in WT and MT-null mice. Liver sections were stained with H & E and scored semiquantitatively for liver injury as described. Cadmium administration resulted in congestion and multifocal hepatic necrosis 16 h after 2.5 mg Cd in WT mice with saline pretreatment (Fig. 3A). Cadmium caused more severe hepatotoxicity 16 h after 2.5 mg Cd in MT-null mice with saline pretreatment (Fig. 3B). In MT-null mice, necrosis was multifocal and consisted of larger lesions with more extensive congestion. Treatment with 60 mg $GdCl_3$ 24 h prior to 2.5 mg Cd completely abrogated the cadmium-induced hepatotoxicity in WT mice (Fig. 3C). In all samples pretreated with 60 mg $GdCl_3$, there was an absence of both congestion and necrosis after Cd administration. A notable histological change is consistent with hydropic change that was not confined to any specific zone of the liver. This hydropic change was not considered deleterious because hydropic change is reversible and there were no increases in serum indices of toxicity. Treatment with 60 mg $GdCl_3$ 24 h prior to 2.5 mg Cd also prevented hepatotoxicity in MT-null mice (Fig. 3D). Pretreatment with $GdCl_3$ protected MT-null mice in a manner that was histologically indistinguishable from the same treatment in WT mice.

Serum indices of Cd-induced hepatotoxicity after $GdCl_3$ pretreatment in WT and MT-null mice. After saline pretreatment, Cd caused marked increases in serum indices of hepatotoxicity in WT mice (Figs. 4A and 4B). Pretreatment with 10 mg $GdCl_3$/kg did not significantly decrease ALT or SDH activities in Cd-treated WT mice. However, pretreatment with 30 and 60 mg $GdCl_3$/kg significantly decreased ALT (95 and 98%, respectively) and SDH (93 and 97%, respectively) activities. In saline-pretreated MT-null mice, Cd administration also caused extensive hepatotoxicity. Pretreatment with 10 mg $GdCl_3$/kg tended to decrease ALT activity (35%) and SDH activity (55%), although these decreases were not significant; whereas pretreatment with 30 mg $GdCl_3$/kg resulted in significantly lower serum ALT (90%) and SDH (85%) activities. Pretreatment with 60 mg $GdCl_3$/kg completely abrogated Cd-induced liver injury in both WT and MT-null mice.

Histological analysis of Cd-induced liver injury and Kupffer cell depletion after $GdCl_3$ administration in WT and MT-null mice. The histological grade of liver injury paralleled the serum markers of hepatotoxicity (Fig. 5A). F4/80-positive Kupffer cells only were noted in liver sections from saline-pretreated mice (Fig. 5B). Compared to WT mice that were administered saline only, saline-pretreated WT and MT-null mice had significantly fewer Kupffer cells (approximately seven per field). In both WT and MT-null mice that were pretreated with all doses of $GdCl_3$ prior to Cd administration, very few F4/80-positive cells were noted.

Hepatic Cd content after $GdCl_3$ and Cd administration. Liver samples were also analyzed for hepatic Cd content (Fig. 6A). Livers from WT mice pretreated with saline, 10, 30, and 60 mg $GdCl_3$/kg prior to 2.5 mg Cd/kg contained 14, 17, 17, and 24 µg Cd/g liver, respectively. Only WT mice pretreated with 60 mg $GdCl_3$/kg prior to 2.5 mg Cd/kg had higher hepatic Cd content than WT mice that were pretreated with saline prior to 2.5 mg Cd/kg. Livers from MT-null mice pretreated with saline, 10, 30, and 60 mg $GdCl_3$/kg prior to 2.5 mg Cd/kg contained 11, 10, 14, and 11 µg Cd/g liver, respectively. $GdCl_3$ pretreatment did not significantly alter Cd accumulation in livers from MT-null mice. Overall, hepatic Cd content was

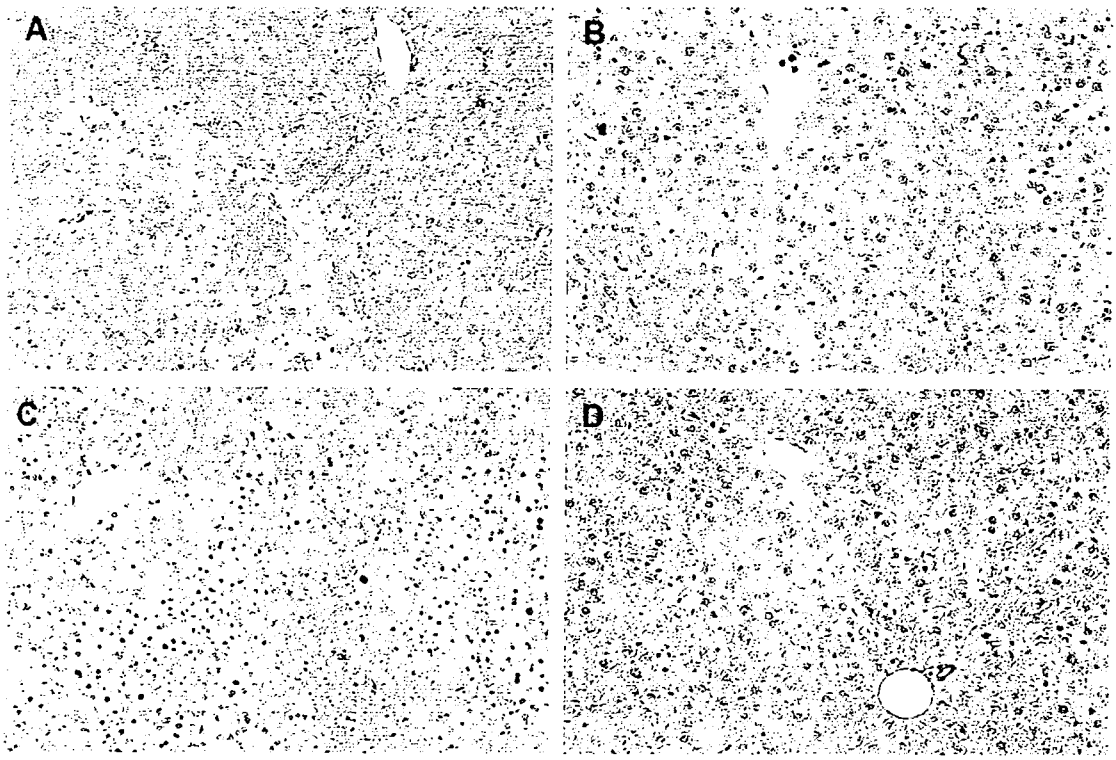

FIG. 3. Photomicrographs showing Cd-induced hepatotoxicity after $GdCl_3$ pretreatment in WT and MT-null mice. Wild-type and MT-null mice were administered saline or various doses of $GdCl_3$ (10, 30, or 60 mg $GdCl_3$/kg) 24 h prior to a hepatotoxic dose of Cd (2.5 mg Cd/kg). Livers were collected 16 h after Cd administration, processed routinely, and stained with hematoxylin and eosin. Photomicrographs (200×) are representative of mouse liver from wild-type (A and B) and MT-null (C and D) mice 24 h after administration of Cd after pretreatment with saline (A and C) or 60 mg $GdCl_3$ (B and D).

slightly higher in WT mice than MT-null mice, although this difference was significant only at 10 and 60 mg $GdCl_3$/kg.

Hepatic MT content after $GdCl_3$ and $CdCl_2$ administration. Hepatic metallothionein was induced in all Cd-treated WT mice (Fig. 6B). Livers from WT mice pretreated with 10 or 30 mg $GdCl_3$/kg had MT concentrations (91 and 100 μg/g liver, respectively) that were similar to saline-pretreated controls (100 μg/g liver), whereas livers from WT mice pretreated with 60 mg $GdCl_3$/kg prior to Cd administration had significantly more MT (115 μg/g liver) than mice administered saline plus 2.5 mg Cd/kg (91 μg/g liver). In MT-null mice, hepatic MT content was not increased and remained unchanged in all treatment groups.

DISCUSSION

Mounting evidence supports the theory that Kupffer cells play a role in Cd-induced hepatotoxicity. Cadmium administration results in an increase in colloidal carbon clearance, indicative of a general increase in phagocytic activity (Sauer *et al.*, 1997; Yamano *et al.*, 2000). Elimination of Kupffer cells with $GdCl_3$ alleviates Cd-induced hepatotoxicity in rats (Sauer *et al.*, 1997; Yamano *et al.*, 1998) but does not protect cultured hepatocytes (Badger *et al.*, 1997). However, the effects of $GdCl_3$ on the liver are not limited to Kupffer cell depletion. For example, $GdCl_3$ has been shown to suppress superoxide production, interfere with inducible nitric oxide synthase expression, and induce MT (Iimuro *et al.*, 1994; Roland *et al.*, 1996; Sauer *et al.*, 1997).

Metallothionein is a low-molecular-weight, cysteine-rich protein that has been shown to be highly inducible and to protect the liver from Cd-induced hepatotoxicity. Cd and other metals, such as zinc, also induce MT, thus conferring resistance to Cd-induced hepatotoxicity (Goering and Klaassen, 1984a,b). This protective effect is absent in MT-null mice, confirming the requirement of MT for protection (Liu *et al.*, 1996). Gadolinium is a pleiotropic metal that, in addition to eliminating Kupffer cells from the liver, induces MT (Sauer *et*

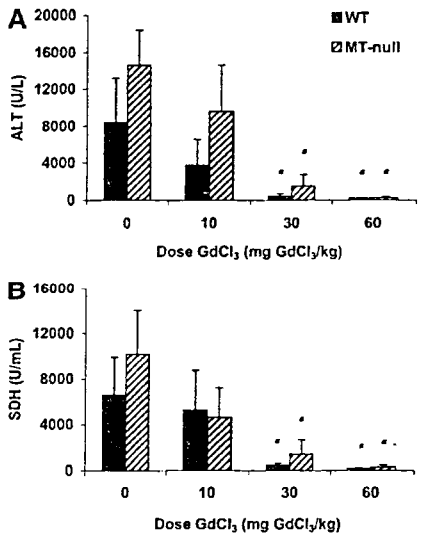

FIG. 4. Assessment of Cd-induced hepatotoxicity after $GdCl_3$ pretreatment in WT and MT-null mice. Wild-type (solid bars) and MT-null mice (hatched bars) were administered saline or various doses of $GdCl_3$ (10, 30, or 60 mg $GdCl_3$) 24 h prior to a hepatotoxic dose of Cd (2.5 mg Cd/kg). Blood was collected 16 h after Cd administration. Hepatotoxicity was assessed by measuring serum indices of hepatotoxicity, namely (A) ALT and (B) SDH activities. *$GdCl_3$-treated mice exhibited significantly less hepatotoxicity than respective saline-treated control mice ($p < 0.05$). Data are presented as means ± SE ($n = 7–8$).

al., 1997). It is therefore reasonable to hypothesize that $GdCl_3$ may increase hepatic MT and thus protect the liver from Cd-induced liver injury.

To determine the dose-dependent effects of $GdCl_3$ on hepatic MT, WT mice were administered various doses of $GdCl_3$. The highest dose of $GdCl_3$ used was 60 mg/kg because higher doses began to cause seizures and death. In these studies, the LD50 was approximately 100 mg $GdCl_3$/kg. All mice survived doses up to 60 mg $GdCl_3$/kg. Twenty-four hours after $GdCl_3$ administration, livers were examined for toxicity, thiol alterations, and depletion of Kupffer cells. Both biochemical and histological examinations were utilized to assess liver injury. Administration of saline, 10, 30, or 60 mg/kg $GdCl_3$ iv did not cause liver injury as measured by quantifying serum indices of hepatotoxicity (data not shown). Livers from both saline- and $GdCl_3$-treated mice were histologically normal, except for some hydropic changes (Figs. 1A and 1B). Kupffer cells were identified immunohistochemically (Figs. 1C and 1D) and found to be eliminated from the liver at all doses of $GdCl_3$ (Fig. 2A). These data indicate that $GdCl_3$ is selectively toxic to Kupffer cells and does not appear to exert toxicity on other cell types of the liver.

Metallothionein and glutathione comprise the primary protein and nonprotein sulfhydryls that participate in hepatoprotection. A wide variety of chemicals, including metals such as Cd and Zn, are known to induce MT. In this study, $GdCl_3$ also increased hepatic MT in a dose-dependent manner (Fig. 2B). Although $GdCl_3$ induced MT significantly, it was neither as potent or effective as Zn or Cd at inducing MT. However, the degree to which $GdCl_3$ induced MT is enough to protect liver from Cd-induced liver injury. Nonprotein sulfhydryls were also examined and were found to be unaffected by $GdCl_3$ treatment (Fig. 2C). Therefore, it appears that, although glutathione is not altered by $GdCl_3$, MT induction may account for the hepatoprotective effects of $GdCl_3$.

While it is not a novel finding that $GdCl_3$ is effective at depleting Kupffer cells from the liver, it is interesting that lower doses of $GdCl_3$ (10 mg $GdCl_3$) are required to deplete Kupffer cells than to induce MT (30 and 60 mg $GdCl_3$/kg) in WT mice. This dose-related difference can be exploited to discern the mechanism of $GdCl_3$-induced hepatoprotection. Therefore, Cd-induced hepatotoxicity was examined in mice after pretreatment with saline or $GdCl_3$. This experiment was conducted in both WT and MT-null mice to eliminate MT as an experimental variable, in addition to comparing the dose re-

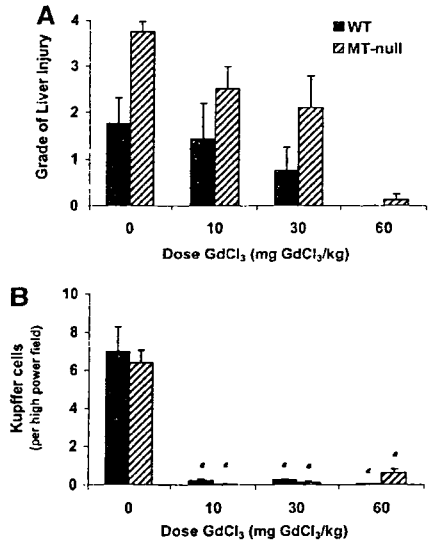

FIG. 5. Histological analysis of Cd-induced liver injury and Kupffer cell depletion after $GdCl_3$ pretreatment. Wild-type (solid bars) and MT-null mice (hatched bars) were administered saline or various doses of $GdCl_3$ (10, 30, or 60 mg $GdCl_3$) 24 h prior to a hepatotoxic dose of Cd (2.5 mg Cd/kg). Livers were collected 16 h after Cd administration and prepared as described under Materials and Methods. (A) Liver sections were visualized with standard hematoxylin and eosin and then scored semiquantitatively for liver injury. (B) Kupffer cells were identified using a primary antibody to the F4/80 macrophage surface antigen (Serotec) and quantified as the number of F4/80-positive cells per high-power field (400×). *Livers from $GdCl_3$-treated mice contained significantly fewer Kupffer cells than livers from saline-treated control mice ($p < 0.05$). Data are presented as means ± SE ($n = 7–8$).

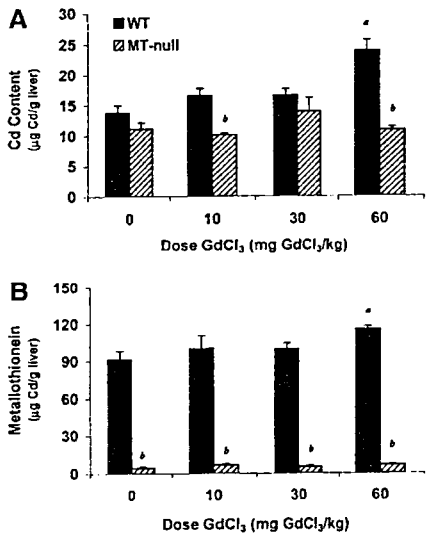

FIG. 6. Assessment of hepatic Cd and MT content after $GdCl_3$ and $CdCl_2$ administration. WT (solid bars) and MT-null mice (hatched bars) were administered saline or various doses of $GdCl_3$ (10, 30, or 60 mg $GdCl_3$) 24 h prior to a hepatotoxic dose of Cd (5 mg Cd/kg). Livers were collected 16 h after Cd administration. Liver samples were prepared and analyzed for Cd (A) and MT (B) content as described under Materials and Methods. [a]Livers from $GdCl_3$-treated mice contained significantly greater Cd or MT than livers from saline-treated control mice ($p < 0.05$). [b]Livers from MT-null mice contained significantly less Cd or MT than livers from WT mice after similar chemical treatment ($p < 0.05$). Data are presented as means ± SE ($n = 7–8$).

sponses of hepatotoxicity, MT induction, and Kupffer cell depletion. Wild-type and MT-null mice were pretreated with saline or the same doses of $GdCl_3$. Subsequently, all mice were administered a hepatotoxic dose of Cd (2.5 mg Cd/kg). Serum indices of hepatotoxicity (Figs. 4A and 4B) were confirmed by histological evaluation (Fig. 5A). After saline pretreatment, Cd caused extensive liver injury in both WT and MT-null mice. Pretreatment of WT and MT-null mice with 30 mg $GdCl_3$/kg 24 h prior to the hepatotoxic dose of Cd alleviated most of the Cd-induced hepatotoxicity. Administration of 60 mg $GdCl_3$/kg effectively abrogated Cd-induced hepatotoxicity in both WT and MT-null mice. These data demonstrate that, in WT mice, $GdCl_3$ induces MT in a dose-dependent manner that is consistent with hepatoprotection. Similarly, MT-null mice also benefit from the hepatoprotective effects of $GdCl_3$. Therefore, while $GdCl_3$ induces MT in WT mice, $GdCl_3$ does not prevent Cd-induced hepatotoxicity by inducing MT.

Kupffer cells were identified immunohistochemically and counted in an effort to compare the dose responses of Kupffer cell depletion and hepatoprotection. Similar numbers of Kupffer cells were present in WT and MT-null mice after saline pretreatment (Fig. 5B). Again, few Kupffer cells could be identified in $GdCl_3$-pretreated WT and MT-null mice. However, the dose-dependent nature of the decrease in Kupffer cells is not consistent with the dose-dependent protection from Cd-induced hepatotoxicity, suggesting that $GdCl_3$ exerts hepatoprotective effects aside from depleting Kupffer cells. The disparity between dose–response relationships of hepatoprotection and Kupffer cell depletion does not conclusively rule out the involvement of Kupffer cells in the mechanism of Cd-induced hepatotoxicity. However, this is strong evidence that there are other changes in the liver that may account for the hepatoprotective effects of $GdCl_3$.

Hepatic Cd content was also quantified to determine whether $GdCl_3$ prevents hepatotoxicity by decreasing the distribution of Cd to the liver (Fig. 6A). In WT mice, the hepatic Cd burden was not decreased with increasing doses of $GdCl_3$. Rather, hepatic Cd content was actually slightly increased (1.7-fold) at 60 mg $GdCl_3$/kg despite decreased toxicity. This is most likely due to sequestration of Cd by $GdCl_3$-induced presynthesized MT. From these data, it can be concluded that $GdCl_3$ pretreatment does not prevent Cd-induced liver injury by decreasing the absolute quantity of Cd in the liver.

Hepatic MT protein levels were examined in WT and MT-null mice to determine whether MT induction accounts for the increased hepatic Cd content as well as to confirm that MT-null mice are truly deficient in MT. Compared to saline-treated WT mice, hepatic MT was greatly increased after saline or $GdCl_3$ pretreatment with subsequent Cd administration (Fig. 6B). At the highest dose of $GdCl_3$ (60 mg/kg), MT was significantly higher than saline pretreatment, indicating that the MT induction by $GdCl_3$ occurs in addition to Cd-induced MT induction. This pattern is consistent with the increases in hepatic Cd, suggesting that the increases in Cd content are due to sequestration of Cd by presynthesized MT. Hepatic MT was absent in MT-null mice, confirming the genotype of the MT-null mice. This same pattern of hepatoprotection was achieved by pretreatment with subtoxic doses of Zn (Liu *et al.*, 1996). However, the current study contrasts previous studies in which Zn pretreatment did not protect against Cd-induced hepatotoxicity in MT-null mice. In this study, MT-null mice are also protected at the same doses of $GdCl_3$ that protect WT mice. Therefore, it can be concluded that MT does not account for the hepatoprotective effects of $GdCl_3$.

Taken together, these data suggest multiple mechanisms for $GdCl_3$-induced protection from Cd-induced liver injury. These data demonstrate that $GdCl_3$ is an effective inducer of MT at doses that are consistent with protection against Cd-induced liver injury. However, MT induction has a dispensable cytoprotective effect as seen in MT-null mice. Additionally, $GdCl_3$ does not alleviate hepatotoxicity by decreasing total Cd accumulation in the liver. Kupffer cells are effectively depleted at doses below those required to prevent Cd-induced liver injury. This does not rule out Kupffer cells in the mechanism of Cd-induced hepatotoxicity, but it does suggest that $GdCl_3$ exerts hepatoprotective effects in addition to depleting Kupffer cells. In summary, this study substantially rules out MT induc-

tion in the mechanism of $GdCl_3$-induced protection from Cd-induced hepatotoxicity.

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