Susceptibility of MT-Null Mice to Chronic CdCl₂-Induced Nephrotoxicity Indicates That Renal Injury Is Not Mediated by the CdMT Complex

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Cadmium (Cd) ranks sixth among the most important toxic chemicals, according to the Agency for Toxic Substances and Disease Registry priority list. Cd is widely used in industry for batteries, electroplating, plastic stabilizers, and pigments, as well as in agriculture as a contaminant in phosphate fertilizer. Cd is nonbiodegradable and environmental Cd levels are increasing. Cd is readily taken up by plants, and the major route of exposure for the general population is from Cd-contaminated food or smoking of Cd-contaminated tobacco. Inhalation of Cd dusts and fumes is an occupational hazard in some industries (Friberg et al., 1986; Goyer et al., 1995).

Chronic human exposure to Cd results in various maladies; the well-known Itai-itai disease is characterized by renal dysfunction, anemia, and osteomalacia combined with osteoporosis and intolerable pain (Friberg et al., 1986). Cd-induced nephrotoxicity is the most important and the most frequently occurring ailment in humans chronically exposed to Cd (Goyer et al., 1995; Goyer and Cherian, 1995).

Cd-induced nephrotoxicity is thought to be dependent on the chemical form of the metal. A single injection of Cd–metallothionein complex (CdMT) produces kidney injury, whereas a single injection of CdCl₂, even at a 10 times higher dose than CdMT, produces hepatotoxicity instead of nephrotoxicity (Nordberg et al., 1975; Min et al., 1986; Dorian et al., 1995; Liu et al., 1996b). Therefore, it was hypothesized that CdCl₂-induced renal injury is mediated by the CdMT complex, which is formed in liver, released into the circulation, and taken up by the kidney, resulting in toxicity (Dudley et al., 1985; Chan et al., 1993). Thus, the CdMT complex, instead of CdCl₂, has been used as a model to study Cd nephropathy for many years. If this scenario is correct, then MT-null mice, which cannot form CdMT, should not develop nephrotoxicity. This hypothesis will be tested in this study.

Most of the total body burden of Cd is associated with metallothionein (MT). MT is a low-molecular weight, cysteine-rich protein (Kägi, 1993). These cysteines bind and store Zn, but can also bind and “detoxify” Cd. It is well accepted that MT is responsible for the detoxification of Cd in many species. Treatment with nontoxic doses of Cd or Zn,

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which increase tissue MT levels, produces tolerance to the toxic effects of a subsequent lethal dose of Cd (Goering and Klaassen, 1983; Goering et al., 1995). This tolerance (i.e., resistance) appears to be due to intracellular sequestration of Cd into an "inert" CdMT, thus reducing the interaction of Cd with target molecules (Goering et al., 1995). Recent studies have shown that MT-transgenic mice are tolerant to Cd lethality and hepatotoxicity (Liu et al., 1995), while MT-I/II null mice are sensitive to Cd lethality and hepatotoxicity (Michalska and Choo, 1993; Masters et al., 1994; Liu et al., 1996b). Thus, one can definitively conclude that both constitutive and induced hepatic MT levels are important in protecting against acute CdCl₂-induced lethality and hepatotoxicity (Klaassen and Liu, 1998).

However, it is not known whether MT can provide long-term protection against Cd toxicity, as the reported protective effects of MT in animals and cell cultures have been done in short-term studies (Petering and Fowler, 1986; Cherian, 1995). With long-term, low-level exposure to Cd, the capacity of MT in the liver to sequester Cd can be exceeded, which leads to liver damage and subsequent release of CdMT into the circulation. The CdMT is considered toxicologically "inert" when stored intracellularly; however, it becomes a potent nephrotoxicant after reaching the systemic circulation (Cherian et al., 1976; Klaassen, 1996). There is little or no information on the role of intracellular MT in chronic Cd nephrotoxicity. Therefore, the second goal of this study is to use the MT-null mouse model to critically determine whether MT protects against chronic Cd-induced renal injury.

**MATERIALS AND METHODS**

**Chemicals.** CdCl₂ was obtained from Fisher Scientific Co. (Fair Lawn, NJ). CdCl₂ was prepared for injection by dissolving it in 0.9% NaCl. ¹⁰⁹/CdCl₂ (5.12 mCi/mg) was obtained from New England Nuclear (Boston, MA). N-Acetyl-β-D-glucosaminidase kits were purchased from Boehringer (Indianapolis, IN). γ-glutamyltransferase, creatinine, glucose, and blood urea nitrogen kits were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

**Animals.** Homozygous MT-I and -II knock-out mice (129/Sv background; Masters et al., 1994) were obtained from Jackson Laboratories (Bar Harbor, ME). The homozygous mutants were mated inter se to maintain the line. Mice were housed in AAALAC accredited rooms with a 12-h light/dark cycle at 70 ± 2°F. Mice were allowed free access to mouse chow (Harian Teklad 7001, Madison, WI) and tap water. Both male and female mice were used, and the results were similar (data not shown).

**Statistics.** Data are expressed as means ± standard error. Comparisons between control and MT-null mice were performed by the Student’s t-test. Significance was set at p < 0.05.

**RESULTS**

Chronic administration of CdCl₂ resulted in dose-dependent lethality and loss of body weight in both control and MT-null mice (Fig. 1). MT-null mice could not survive the daily dose of 0.4 mg Cd/kg sc for 3 weeks (18 infections) or the daily dose of 0.2 mg Cd/kg for 6 weeks (36 infections; 40% mice died at this time). In contrast, control mice survived daily doses of 2.4 mg Cd/kg for 4 weeks or 1.6 mg Cd/kg for 6 weeks. During the 10-week exposure period, no body weight gain was observed in MT-null mice receiving 0.1 mg Cd/kg, while a dose of 0.8 mg Cd/kg was required to produce similar effect in control mice. Thus, MT-null mice were approximately eight times more susceptible than control mice to chronic Cd toxicity.

The major target organ for chronic Cd toxicity is the kidney, and it was critical to examine the renal Cd concentrations during chronic exposure to Cd. Cd accumulation in kidney was dose-dependent (Fig. 2, left). Maximum renal Cd concentrations (>110 μg Cd/g kidney) occurred in mice receiving daily doses higher than 0.8 mg Cd/kg. In comparison, in MT-null mice receiving daily Cd at the maximum tolerated doses, the renal Cd concentration did not exceed 10 μg/g kidney. Renal Cd accumulation was also time-dependent. For example, at a daily dose of 0.1 mg Cd/kg, renal Cd concentrations in control mice were 16, 34, and 64 μg/g kidney after 3, 6, and 10 weeks of Cd injection, respectively. In contrast, in MT-null mice
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FIG. 1. Changes in body weight following chronic CdCl₂ administration to control (0.05 to 2.4 mg Cd/kg) and MT-null mice (0.0125 to 0.4 mg Cd/kg). Mice received daily doses of CdCl₂ sc, six times/week for up to 10 weeks. Data are means of six to eight mice.

receiving the same dose of Cd, renal Cd concentration was only about 7-10 μg/g kidney throughout the 10-week period. Renal Cd-binding protein (MT) concentrations are shown in

FIG. 2. Renal Cd and MT concentrations following chronic CdCl₂ administration to control (0.05 to 2.4 mg Cd/kg, sc, six times/week) and MT-null mice (0.0125 to 0.4 mg Cd/kg, sc) for 3, 6, and 10 weeks. Data are means ± SE of six to eight mice. *Significantly different from controls (p < 0.05).

the right panel of Fig. 2. Renal MT was induced up to 150-fold in control mice, reaching 800 μg MT/g kidney at 10 weeks of exposure. In contrast, renal Cd-binding protein concentrations (MT equivalents) in MT-null mice were at background levels and remained unaltered during the 10-weeks of Cd exposure.

Urinary protein and glucose are commonly used indices for proximal tubular injury. At 3 and 6 weeks of Cd exposure, MT-null mice excreted more urinary protein and glucose than controls at doses of 0.1 mg Cd/kg and higher (Fig. 3). It is interesting to note that after 10 weeks of multiple injections of CdCl₂ (60X), urinary protein and glucose excretion do not appear to be sensitive indices for evaluating nephrotoxicity, as they were not higher than the levels observed at 3 and 6 weeks of Cd exposure.

Urinary excretion of γ-glutamyltransferase and N-acetyl-β-D-glucosaminidase, two enzymes whose urinary excretion is associated with proximal tubular cell damage, were also examined (Fig. 4). In general, increases in these two enzymes paralleled chronic CdCl₂-induced renal morphological changes. MT-null mice excreted more of these enzymes into the urine than did control mice throughout the 10-week period of Cd exposure.

Chronic administration of CdCl₂ enlarged the kidneys in a time- and dose-dependent manner, with up to 50% increase in kidney/body weight ratios in both control and MT-null mice (Fig. 5, left). MT-null mice had significantly higher kidney/body weight ratios than controls at all comparable doses. Blood urea nitrogen (BUN) concentrations were also elevated dose-dependently by repeated CdCl₂ administration in both control and MT-null mice (Fig. 5, right). At doses of 0.1 to 0.2 mg
Cadmium Chloride (mg Cd/kg, sc)

FIG. 4. Urinary excretion of γ-glutamyltransferase and N-acetyl-β-D-glucosaminidase following chronic CdCl₂ administration to control (0.05 to 2.4 mg Cd/kg, sc) and MT-null mice (CdCl₂ 0.0125 to 0.4 mg Cd/kg, sc) daily for 3, 6, and 10 weeks. Data are means ± SE of six to eight mice. *Significantly different from controls (p < 0.05).

Cd/kg, MT-null mice had higher BUN levels than control mice, indicating more renal damage.

The most important method for evaluating chronic CdCl₂-induced nephrotoxicity is histopathology. Chronic Cd exposure produced damage to the entire kidney, including tubular degeneration, tubular cell apoptosis, interstitial inflammation, and glomerular cell proliferation and swelling (Groten et al., 1994; Hiratsuka et al., 1996; Liu et al., 1998a). These pathological lesions progressed with time, with more lesions being observed after 10 weeks of exposure. At the dose of 0.1 mg Cd/kg, MT-null mice (Fig. 6B) were more susceptible than control mice (Fig. 6A) to chronic Cd-induced renal lesions, including tubular degeneration, vacuolation, and tubular cell apoptosis. The glomeruli of the MT-null mice were more markedly swollen as compared to control mice.

FIG. 5. Kidney/body weight ratios and blood urea nitrogen concentrations following chronic CdCl₂ administration to control (0.05 to 2.4 mg Cd/kg, sc) and MT-null mice (0.0125 to 0.4 mg Cd/kg, sc) daily for 3, 6, and 10 weeks. Data are means ± SE of six to eight mice. *Significantly different from controls (p < 0.05).

DISCUSSION

The present study has demonstrated that chronic CdCl₂ produced renal injury in MT-null mice. We originally hypothesized that MT-null mice would be resistant to Cd nephrotoxicity, because these mice cannot synthesize the CdMT complex in response to Cd exposure. Contrary to our expectation, MT-
null mice were more susceptible than control mice to chronic Cd-induced renal injury. This is the first study to clearly indicate that Cd-induced renal injury is not mediated via the CdMT complex. The possibility that Cd may bind to other low-molecular-weight proteins or thiols (such as glutathione and cysteine) for its delivery to the proximal tubular cells requires further investigation.

Cd ion has been shown to be directly toxic to renal cells, producing effects such as inhibition of Na\(^+\)/glucose cotransport in renal cortical cells (Blumenthal et al., 1990), disruption of intracellular junctions and actin filaments in LLC-PK1 cells (Prozialeck et al., 1993), interference with DNA and protein synthesis in mesangial cells (Chin and Templeton, 1992), and increases in LDH leakage from primary cultures of renal tubules (Groten et al., 1994). In hamsters, inorganic Cd salts also produce acute renal injury (Rhem and Waalkes, 1990). These observations further support the finding that Cd-induced toxicity to kidney cells is not necessarily mediated via the CdMT complex.

Whether induction of MT is beneficial or harmful for the kidney during chronic Cd exposure has been an issue of debate for many years. Induction of MT protects liver from acute Cd toxicity by sequestering the metal in the cytosol, but the formed CdMT complex is a potent nephrotoxicant when it is released into the circulation (Nordberg et al., 1975; Cherian et al., 1976; Squibb et al., 1984; Maitani et al., 1988; Klaassen and Liu, 1997). The results from our recent studies indicate that the current theory for Cd nephropathy is incomplete for several reasons including: (1) renal Cd burden does not come only from CdMT, but also from inorganic Cd salts (Dorian et al., 1995; Liu et al., 1996a); (2) a single injection of CdMT does not mimic chronic Cd nephropathy (Liu et al., 1998a); (3) chronic CdMT administration produces renal injury in a manner similar to chronic CdCl\(_2\) administration (Groten et al., 1994; Min et al., 1996; Liu et al., 1998a), but differs from a single injection of CdMT; and (4) intracellular MT protects against chronic CdMT-induced renal injury (Liu et al., 1998b), but does not play a major role in acute CdMT nephrotoxicity (Liu et al., 1996b,c).

The present study clearly demonstrates that MT serves to protect against chronic Cd toxicity. MT-null mice were approximately eight times more susceptible to chronic Cd-induced lethality and nephrotoxicity. These results have clearly demonstrated for the first time that intracellular MT not only protects against acute Cd toxicity but also protects against chronic Cd toxicity. It should also be pointed out that these MT-null mice are also more sensitive to control mice to Cd-induced chronic hepatoxicity (Habeebu et al., 1998a), immunotoxicity (Liu et al., 1998c), and osteotoxicities (Habeebu et al., 1998b). Thus, MT is an important cellular protein in protecting Cd toxicity, either from acute or from chronic exposure.

The reason why MT-null mice have an increased susceptibility to Cd-induced nephrotoxicity is not completely understood, but could result from several factors: First, the lack of MT eliminates the major protective mechanism to sequestrate Cd from critical target organelles. It is free, non-MT-bound Cd, rather than "total Cd" that produces cell injury (Goyer et al., 1989). Additionally, Cd-induced renal damage may result from inflammation (Weiss et al., 1994) and oxidative stress (Bagchi et al., 1997; Zaman and Shaikh, 1997). MT has also been proposed to play an important role in protecting against oxidative stress (Sato and Brenner, 1993), and thus deficiency in MT renders the kidney of MT-null mice more susceptible to oxidative damage, as seen in the liver (Zheng et al., 1996).

It is also important to point out that MT-null mice were more susceptible than controls to chronic CdCl\(_2\)-induced apoptosis, as evidenced by condensed nuclei, shrinkage of cells, and increased eosinophilia (as judged by H&E stain). Apoptosis is a controlled form of cell death that serves as a molecular point of regulation for biological processes. Because the renal apoptotic cells contain high Cd content (Tanimoto et al., 1993), apoptosis is thought to be a mode for elimination of Cd and critically damaged cells from kidneys, thus preventing the disturbance of tissue structure and integrity of the kidney. In chronic Cd-intoxicated animals, necrosis is rare, but apoptosis is common (Hamada et al., 1991; Tanimoto et al., 1993, Liu et al., 1998a; Habeebu et al., 1998a). Enhanced apoptosis was reported in MT-null cells or animals exposed to oxidative stress (Kondo et al., 1997), cisplatin (Kondo et al., 1997; Liu et al., 1998e), and chronic CdMT (Liu et al., 1998b), supporting the notion that lack of MT results in an increase in Cd-induced apoptosis.

Other pathological lesions observed in the kidney includes tubular degeneration, glomerular cell proliferation and swelling, interstitial nephritis, and interstitial fibrosis. These lesions were dose- and time-dependent. Although the nature and type of lesions were the same in both control and MT-null mice, it took approximately eight times more Cd to produce a similar extent of injury in control mice than in MT-null mice (0.8 vs 0.1 mg Cd/kg).

In conclusion, the present study using MT-null mice has demonstrated that the renal injury produced by repeated Cd administration is not necessarily mediated via the CdMT complex and that MT is an important intracellular protein protecting against chronic Cd nephropathy.

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