Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 584–588, January 1994
Biochemistry

Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium

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ABSTRACT **We inactivated the mouse metallothionein (MT)-I and MT-II genes in embryonic stem cells and generated mice homozygous for these mutant alleles. These mice were viable and reproduced normally when reared under normal laboratory conditions. They were, however, more susceptible to hepatic poisoning by cadmium. This proves that these widely expressed MTs are not essential for development but that they do protect against cadmium toxicity. These mice provide a means for testing other proposed functions of MT *in vivo*.**

Metallothioneins (MTs) were identified by Margoshes and Vallee (1); since then MTs have been described in most vertebrates and in a wide variety of invertebrate species (for review, see refs. 2 and 3). MTs are characterized by their low molecular weight, high cysteine content (≈30%), lack of aromatic residues, and the presence of 7–12 metal atoms per molecule (2). In mammals, the cysteine residues are absolutely conserved in number and serve to coordinate heavy metal atoms such as zinc, copper, and cadmium via mercaptide linkages. Two major isoforms of MT have been described in mammals, designated MT-I and MT-II, that can be resolved by ion-exchange chromatography. In the mouse, the genes encoding these two isoforms are ≈6 kb apart on chromosome 8 (4). MT-I and MT-II are coordinately regulated in the mouse and the proteins are thought to be functionally equivalent (2, 4). It is now known that there are two other members of the MT gene family (at least in humans and mice) that we refer to as MT-III and MT-IV (5). Unlike MT-I and MT-II, which are expressed in most organs, MT-III expression appears to be restricted to the brain (6) and MT-IV is only expressed in certain stratified squamous epithelia (unpublished observation).

MTs have been postulated to detoxify metals, play a role in zinc and copper homeostasis during development, regulate synthesis, assembly, or activity of zinc metalloproteins, and protect against reactive oxygen species (for review, see refs. 2 and 3). To examine these proposed functions *in vivo*, we inactivated both alleles of the ubiquitously expressed MT-I and MT-II genes.

MATERIALS AND METHODS

Construction of the Disruption Vector. To disrupt the MT-II gene, oligonucleotides 52 (5′-GATCCACGGTTGACTAAGCTA-3′) and 53 (5′-GATCTAGCTTAGTCAACGTG-3′) were annealed and then ligated into the *Bam*HI site of pKH, a plasmid that contains both MT-I and MT-II sequences (4). The MT-I gene was inactivated by two oligonucleotide insertions; one in the *Age* I site of exon 1 and the other in the *Sst* II site of exon 3. For exon 1, oligonucleotide 315 (5′-CCGGATAGGTACCAATTAT-3′) was annealed to oligonucleotide 316 (5′-CCGGATAATTGGTACCTAT-3′); for exon 3, oligonucleotides 193 (5′-GATGTAACGGTCACATCACCGC-3′) and 192 (5-′GGTGATGTGACCGTTACATCGC-3′) were annealed. The 1.9-kb polNeo gene (7) was inserted between the MT-I and MT-II genes by replacing an internal 2-kb *Stu* I fragment. This construct was inserted into pBX-TK, a plasmid that contains the herpes simplex virus thymidine kinase gene.

Generation of Mice with Disrupted MT-I/MT-II Genes. The disruption construct was linearized within the pBX vector and then 20 μg of DNA was electroporated into AB1 embryonic stem cells (8). The stably transfected cells were selected by culturing with G418 (300 μg/ml) and ganciclovir (2 μg/ml). The clones were screened by PCR utilizing primers 194 (5′-CGGGGCACCGCAAAGGGCGC-3′) plus 200 (5′-CTAGCTTAGTCAACCGTGGATCCATCTA-3′) for MT-II and 193 (see above) plus 195 (5′-GCTGTTTTCCATGAGGAAGCTTATG-3′) for MT-I in the buffer described by Soriano *et al.* (7). PCR-positive clones were expanded and analyzed on a Southern blot. The positive clones were injected into C57BL/6 blastocysts and transplanted into pseudo-pregnant females (8). The resultant chimeric mice were bred to 129/SvCPJ mice and heterozygous males and females were then bred to yield mice homozygous for both of the disrupted MT alleles. In all subsequent studies described here, the animals were only mated with the 129/SvCPJ strain of mice to maintain the genetic background.

Analysis of MT Transcripts. Mice were given 25 mM $ZnSO_4$ in their drinking water ad libitum for 7–14 days to induce MT gene transcription and then were sacrificed. The liver was removed and total nucleic acids were isolated (6). For reverse transcription (RT)–PCR, 1 μg of total nucleic acids was digested with RNase-free DNase I for 20 min at 25°C. Then proteinase K and oligo (dT) were added, and the reaction mixture was incubated at 45°C for 20 min, 10 min at 95°C, and then 20 min at 23°C. Then 4 μl of reverse transcriptase mixture containing all four dNTPs (each at 1.25 mM) and 4 units of avian myeloblastosis virus reverse transcriptase was added, incubated at 37°C for 30 min, and then heated at 95°C for 5 min. The transcripts were amplified by adding 2 μl of the cDNA to a 30-μl PCR mixture. The PCR primers used for MT-I amplification were primers 408 (5′CAGCAGGAGCAGCAGCTCTTCTTGCAG-3′), which spans intron 2, and 344 (5′-CACGACTTCAACGTCC-3′), which is located in the 5′ untranslated region of MT-I mRNA. Oligonucleotide 408 was also used for MT-II with oligonucleotide 409 (5′-GCTCCTAGAACTCTTCAAAC-3′), which is located in the 5′ untranslated region of MT-II mRNA.

Cadmium Exchange, Chromatography of MT Protein, and Metal Analysis. For quantitation of MT protein in various tissues, a 5% homogenate was prepared in 20 mM Tris·HCl

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Abbreviations: MT, metallothionein; SGPT, serum glutamic-pyruvic transaminase; SGOT, serum glutamic-oxaloacetic transaminase; RT, reverse transcription.

(pH 8), and 700 μl was mixed with 25 nmol of $CdSO_4$ containing 2.5 μCi of carrier-free ^{109}Cd (New England Nuclear; 1 Ci = 37 GBq) and equilibrated at room temperature for 20 min. The sample was next placed in a boiling water bath for 1.5 min and then centrifuged at 14,000 rpm for 5 min in a microcentrifuge. The supernatant was loaded onto a 1 × 40 cm Sephadex G-75 column equilibrated with 20 mM Tris·HCl, pH 8/50 mM NaCl; 0.85-ml fractions were collected and assayed for radioactivity. Metal content was determined by inductively coupled plasma emission spectroscopy using a Jarrel Ash 955 spectrophotometer. Samples from Sephadex G-75 were used without further treatment. Tissue samples were digested to completion in concentrated HNO_3, evaporated to dryness, and resuspended in 2.5 mM HNO_3.

Cadmium Toxicity. A dosage of cadmium [10 μmol per kg (body weight) per day] that was sublethal for normal rodents was chosen (9). Approximately 200 μl of 1 mM $CdSO_4$ in H_2O was injected subcutaneously. Blood was either collected by cardiac puncture or orbital eye bleed. Samples were allowed to clot and then separated by microcentrifugation. Sera were submitted to a veterinary service laboratory (Phoenix Central Laboratory, Woodinville, WA) for a complete serum chemistry screen. Organs of interest were fixed in 10% (vol/vol) formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

RESULTS

Strategy for MT-I and MT-II Disruption. The murine MT-I and MT-II locus is depicted in Fig. 1*A*. In the disruption vector shown in Fig. 1*B*, a 2-kb *Stu* I segment between the MT-I and MT-II genes was replaced with the polNeo gene for positive selection and a thymidine kinase gene was included for negative selection. To disrupt the MT-I and MT-II genes, oligonucleotide pairs that encode in-frame stop codons were inserted into convenient restriction sites in the exons of the two genes. The disruption vector was transfected into AB1 cells, which were selected for resistance to G418 and ganciclovir. Clones were subjected to PCR analysis with primers a and b, specific for MT-II, and primers c and d, specific for MT-I (Fig. 1 *A* and *B*). The resulting products, if a correct targeting event occurred, should be 730 and 350 bp for MT-II and MT-I, respectively. Correct clones, as identified by PCR, were expanded and subjected to Southern blot analysis. These screens identified four clones in which both MT-I and MT-II alleles were disrupted, four clones in which only the MT-I gene was disrupted, and one clone in which only MT-II was disrupted. Three of the clones with both MT alleles disrupted were injected into blastocysts and one gave germ-like transmission of the mutant alleles.

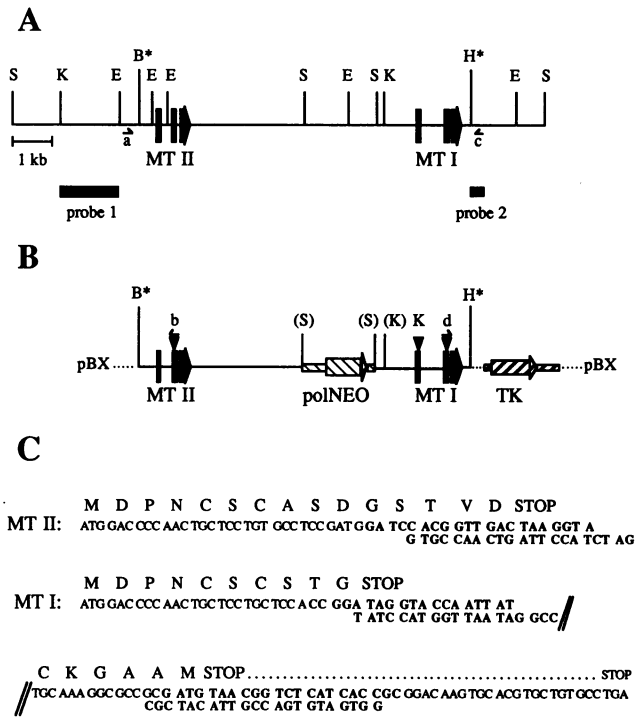

FIG. 1. Murine MT-I/MT-II locus and disruption strategy. (*A*) The MT-I and MT-II locus. All *Eco*RI (E), *Kpn* I (K), and *Stu* I (S) restriction sites and the probes used in Southern blot analysis are shown. The *Bss*HII (B*) and *Hin*dIII (H*) sites represent the boundaries of homology. Locations of the PCR primers used to identify homologous recombinants are indicated by lowercase type and arrows (a, primer 194; b, primer 200; c, primer 195; d, primer 193). (*B*) The disruption construct. (*C*) The sequence of the oligonucleotide insertions, in boldface type, and the location of the in-frame stop codons.

Mice heterozygous (+/−) for the mutant alleles were detected by DNA dot hybridization using a probe from the neomycin-resistance gene. These mice were inbred to generate homozygotes (−/−), which were initially detected by quantitative dot hybridization and confirmed by Southern blot analysis. Fig. 2*A* shows a Southern blot of DNA that had been digested with *Kpn* I and *Eco*RI and hybridized with probe 2 (Fig. 1*A*) from control, heterozygous, and homozygous mutant mice. DNA from a normal animal (+/+) has a single band at 3.0 kb and a homozygous mutant (−/−) has a single band at 2.1 kb due to the *Kpn* I site from the oligonucleotide insertion in exon 1 of MT-I. Heterozygotes (+/−) display both bands.

MT-I and MT-II Mutant Alleles Are Transcribed but Not Translated. The oligonucleotide insertions should produce transcripts that are longer than normal but nonfunctional due to in-frame stop codons (Fig. 1*C*). Examination of RNA from zinc-induced liver by RT–PCR, using primers that flank the insertion in exon 1 of MT-I or exon 2 of MT-II, should give products of 178 bp and 169 bp for normal MT-I and MT-II, respectively, whereas transcripts with the insertions should be 15–17 nt longer. Fig. 2*B* shows that the mutant alleles are transcribed and that heterozygotes make approximately equal amounts of normal and mutant mRNAs.

To determine whether the oligonucleotide insertions block the synthesis of MT protein, ^{109}Cd exchange followed by

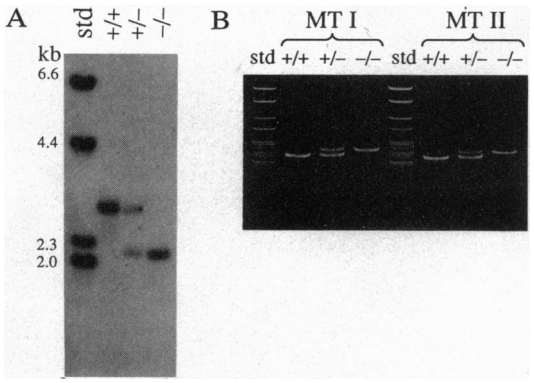

FIG. 2. Southern blot and transcript analysis of MT. (*A*) DNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) MT-null mice was digested to completion with *Eco*RI and *Kpn* I, electrophoresed on a 0.7% agarose gel, transferred to a nitrocellulose membrane, and then screened with probe 2 from Fig. 1*A*. The standard (std) is a *Hin*dIII digest of λ DNA. (*B*) RNA from zinc-induced liver was analyzed by RT–PCR using primers that flank the oligonucleotide insertion sites in exon 1 of MT-I or exon 2 of MT-II. The products were electrophoresed on a 10% polyacrylamide gel and visualized with ethidium bromide. The standard (std) is a *Msp* I digest of pBR322.

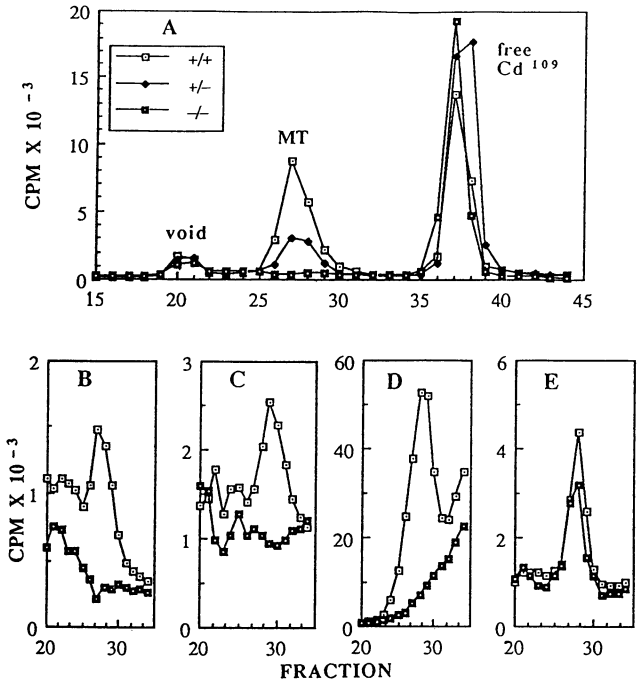

FIG. 3. Tissue survey of MT protein expression by ^{109}Cd exchange and Sephadex G-75 chromatography. All animals were reared with 25 mM $ZnSO_4$ in their drinking water ad libitum to induce MT. (*A*) Expression in liver for wild-type (+/+), heterozygous (+/−), and homozygous (−/−) MT-null mice. The large peak of free ^{109}Cd indicates that the proteins were saturated with metal. MT protein expression in kidney, testis, intestine, and brain (*B–E*, respectively) from normal □ and MT-null ■ mice.

Sephadex G-75 chromatography of the heat-stable material was performed on liver extracts from zinc-induced mice. Fig. 3*A* shows that in normal mice there is a large peak of ^{109}Cd at $V_e/V_o \approx 1.4$, where MT elutes from this column. In heterozygotes this peak is ≈50% that of controls whereas homozygous mutants show no ^{109}Cd binding in this region. Analysis of testis, kidney, and gut by cadmium exchange and G-75 chromatography failed to detect any peak of MT protein in homozygous mutants (Fig. 3 *B–D*). When MT-null brain was assayed, the MT peak was reduced but not eliminated (Fig. 3*E*); the residual MT protein is undoubtedly the brain-specific MT-III isoform.

Analysis of Phenotype. About 125 mutant mice have been maintained for up to 8 months under normal laboratory conditions without any evidence of an abnormal phenotype. Male homozygotes can sire progeny when crossed with female homozygotes and female homozygotes carry their litters to term. The average litter sizes of control and MT-null mice were 4.3 ± 1.2 ($n = 6$) vs. 5.5 ± 1.7 ($n = 8$), respectively.

The ability of MT-null mice to detoxify cadmium was studied by daily injection of 10 μmol of cadmium/kg (body weight). Two groups of normal 129/SvCPJ mice were tested; one group of nine males was treated for 2 days and another group of four males and four females was treated for 14 days. No lethality or other overt physical change was observed in either group. When a group of nine MT-null males was administered the same regimen, seven were dead at 48 hr. The experiment was repeated with seven more males, but it was concluded at 36 hr before any animals died. When a group of seven MT-null females was tested, there was no lethality at 48 hr. However, after 4 days, four had died and the others appeared physically distressed and were killed for analysis. Clearly, the MT-null mice are much more sensitive to the toxic effects of cadmium than control mice and the MT-null females appear to be less affected than males. Sera and several organs were collected from the survivors of each group.

Livers from cadmium-injected MT-null mice exhibited consistent histopathological changes including focal areas of cellular degeneration and necrosis, congestion, and hemorrhaging (Fig. 4*B*). Livers from control mice treated for 2 days appeared normal (Fig. 4*A*). All the control and MT-null males showed signs of extensive testicular necrosis. Kidney and gut from all the experimental groups appeared normal.

The sera from all the mice were submitted for a chemistry screen that included total protein, albumin, globulin, glucose, cholesterol, creatinine, blood urea nitrogen, alkaline phosphatase, serum glutamic-pyruvic transaminase (SGPT), and serum glutamic-oxaloacetic transaminase (SGOT). All values for these markers were within the normal range with the exception of those discussed below.

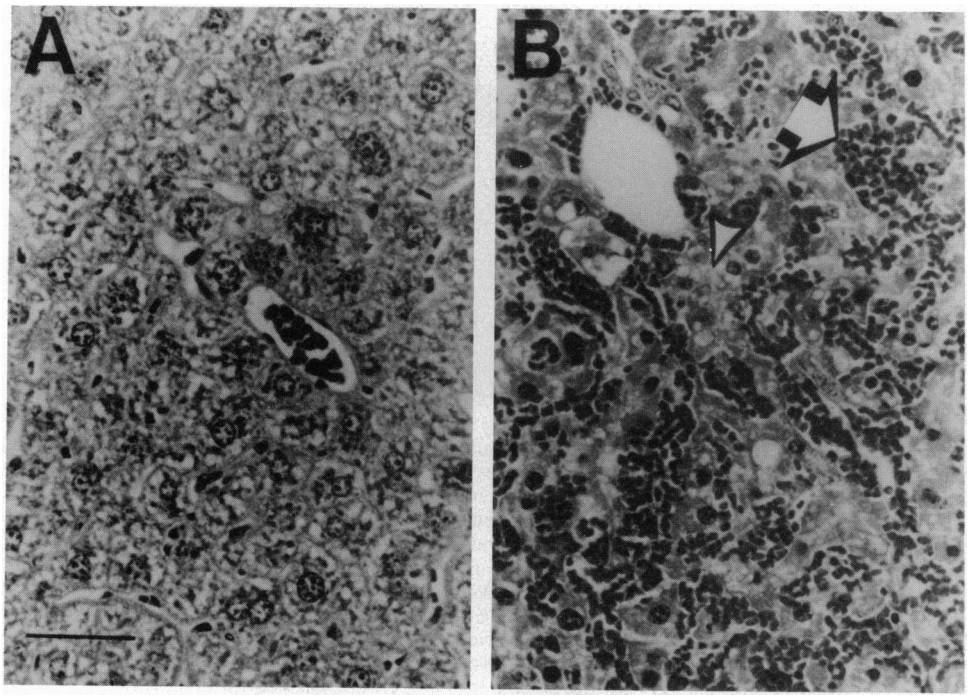

FIG. 4. Histological analysis of cadmium-treated livers. Control liver (*A*) and MT-null liver (*B*) from mice treated for 2 days with cadmium. Hemorrhaging is indicated by the arrow and necrosis is indicated by the arrowhead. (Bar = 100 μm.)

The normal mice that received two doses of cadmium showed a 9-fold elevation in SGPT, likely due to an acute response to metal, which returned to basal levels by 2 weeks (Table 1). SGOT activity also increased at 2 days but the change was less dramatic and did not quite return to basal levels after 2 weeks.

In untreated female MT-null mice, the SGPT and SGOT levels in sera were elevated ≈2-fold compared to controls whereas untreated MT-null males were similar to control animals. The MT-null males showed a large increase in serum SGPT and SGOT after 2 days of cadmium treatment. All mice with serum SGPT values >1400 international units/liter showed clear histological evidence of hemorrhaging with associated hepatocellular degeneration; both males at 48 hr but only three of seven males at 36 hr were clearly affected (Table 1). The female MT-null mice that were treated for 4 days showed similar elevations in SGPT and SGOT activities. The levels for alkaline phosphatase in untreated MT-null mice were higher than controls, but there was no consistent response to cadmium treatment. The serum creatinine and blood urea nitrogen were not elevated in any of the groups examined, indicating that there was no nephrotoxicity.

The amount of cadmium that accumulated in the liver was also determined. In controls, there was 35 ppm of cadmium in the liver at 2 days and 195 ppm at 2 weeks. The MT-null mice accumulated only 15 ppm of cadmium after 2 days (males) or 4 days (females). To determine the distribution of the cadmium, liver extracts of control mice treated for 2 weeks and MT-null mice treated for 2 days were heated, supernatants were chromatographed on Sephadex G-75, and fractions were assayed for metal content. In the control, all of the cadmium was associated with MT, whereas in the MT mutants, none of the cadmium was recovered in the heat-stable fraction, indicating that it was all associated with the heat-insoluble material (data not shown).

DISCUSSION

The widespread inducible expression of MT-I and MT-II during development and in the adult has suggested that these proteins might play an essential role in zinc and copper metabolism during development and provide protection against various environmental stresses. The results of the gene knock-out experiments described here and elsewhere (10) indicate that these MTs are not essential for development or reproduction.

We have examined >125 mice on the inbred 129/SvCPJ genetic background and ≈30 outbred to C57BL/6 mice for up to 8 months of age and observed no overt abnormalities. However, neither a thorough physiological nor histological analysis has been performed. The histology of liver, kidney, intestine, and testis of 6 male MT-null mice reared on Purina mouse chow 5015 appeared normal. However, three of the four livers from MT-null females contained cytoplasmic inclusions. The serum chemistry of these females was notable for moderately elevated SGPT, SGOT, and alkaline phosphatase but, like MT-null males, was otherwise normal for all other markers tested.

The normal development and reproductive function of the MT-null mice are particularly surprising because these MTs are expressed at high levels in germ cells, preimplantation embryos, and the placenta after implantation (11–13). Furthermore, during embryonic development, there is a large increase in MT in the fetal liver between days 12 and 17 that has been postulated to provide a mechanism for sequestering metals from the maternal circulation for subsequent embryonic development (14). The expression of the recently discovered MT-III and MT-IV genes is thought to be restricted to brain and certain squamous epithelia. Neither is expressed in testis or fetal liver, but expression in early embryos and placenta has not been examined. Thus, it is possible that they, or other undiscovered MTs, could be expressed during early developmental stages and compensate for lack of MT-I and MT-II. The chromatographic analysis of cadmium-labeled proteins clearly indicates that there are no compensating MTs in liver, kidney, or intestine, whereas in brain the residual cadmium-binding protein presumably is MT-III.

Several cell lines, including S49, W7, and BHK, fail to express MT due to DNA methylation (5), yet they grow well. Activation of the endogenous MT genes or transfection of MT genes into these cells does not improve their growth characteristics in normal medium (R.D.P., unpublished observations). These results suggest that MTs are not essential for synthesis or function of metalloenzymes or other metalloproteins. These knock-out experiments confirm this result *in vivo*, when mice are reared on a normal diet. It will be important to determine whether MTs are essential when mice are reared on zinc- or copper-deficient diets.

MT was discovered by virtue of its ability to bind cadmium (1). Numerous subsequent studies have documented the ability of cadmium to displace zinc from MT and to induce the synthesis of MT mRNA (for review, see ref. 2). Furthermore, cultured cells can be selected that are resistant to cadmium toxicity, and invariably these cells synthesize more MT due to MT gene amplification (15, 16). This has been the strongest evidence for a function of MT in cadmium detoxification. Cells with amplified MT genes are also more resistant to zinc and copper toxicity (16). When normal mice were injected with 10 μmol of cadmium per kg per day, virtually all the hepatic cadmium was bound to MT. However, most of the

Table 1. Analysis of serum enzymatic markers for signs of hepatotoxicity in response to cadmium

Genotype	Sex	Animals, no.	Days of Cd treatment	Hepatic Cd, μg/g of tissue	Enzyme activity, international units/liter		
					SGPT	SGOT	Alkaline phosphatase
MT +/+	F	4	0	0	77 ± 36	82 ± 22	73 ± 3
MT +/+	M	4	0	ND	45 ± 36	78 ± 21	146 ± 71
MT +/+	M	9	2	35 ± 6	627 ± 319	213 ± 67	91 ± 28
MT +/+	M/F	4 /4	14	195 ± 27	91 ± 61	145 ± 31	157 ± 20
MT −/−	F	4	0	0	162 ± 84	145 ± 64	261 ± 34
MT −/−	M	4	0	ND	38 ± 16	99 ± 29	284 ± 15
MT −/−	M	4	1.5	15 ± 2	397 ± 170	318 ± 78	113 ± 31
	M	3	1.5	18 ± 3	>1400*	>1400*	169 ± 65
MT −/−	M	2	2	12 ± 1	>1400*	>1400*	72 ± 1
MT −/−	F	3	4	15 ± 2	2045 ± 1248	>1400*	408 ± 79

ND, not determined; F, female; M, male. Cadmium concentration is expressed as the mean ± SEM. The values listed for the three enzymes are the mean ± SEM.

*Values were out of the linear range in the first analysis and no more sample was available to get precise readings.

MT-null mice died of a similar regimen between 36 and 96 hr, with the females being more resistant than males. These data confirm the importance of MT-I and/or MT-II for protection against cadmium toxicity *in vivo*.

Death in the MT-null mice is associated with hepatocellular injury. Treatment of neonatal rats with cadmium results in an initial displacement of zinc from endogenous MT and then the induction of more MT (17, 18). However, depending on the dose of cadmium, there can be hepatic damage prior to induction of sufficient MT to bind the incoming cadmium. Although the livers of the control mice appeared normal at both 2 days and 2 weeks, there was apparently some hepatocellular damage at the early time point because there was a transient rise in serum SGPT levels at 2 days. In the MT-null mice, both MT-I and MT-II mRNAs are induced in the liver but intact proteins cannot be synthesized. Consequently, the cadmium binds to other cellular proteins, instead of MT, resulting in their inactivation. The most obvious histological manifestation is the massive infiltration of blood cells between parenchymal cells with signs of hepatocellular necrosis in the most severe cases. The infiltrating blood cells and resultant hemorrhaging into the parenchyma suggests that the hepatic endothelial cells are especially sensitive to cadmium. The vascular endothelial cells, which have been shown to synthesize MT (19), may be the target tissue in acute cadmium poisoning (20). Their breakdown and the resulting hemorrhage could lead to anoxia, hepatocyte death, and the release of SGPT. Alternatively, the primary lesions may reside in hepatocytes and their destruction indirectly results in a breakdown of endothelial cell integrity. The testicular endothelium of both normal and MT-null mice was destroyed by cadmium, suggesting that this sensitivity is unrelated to MT (21). Testicular sensitivity to cadmium is linked to the *cdm* gene, which maps to a different chromosome than MT (22).

There are several genetic conditions, including human Menkes disease, mottled mice, the LEC rat and Bedlington terriers, in which large amounts of copper–MT accumulate in various organs due to defects in copper transport (for review, see ref. 23). Considering that MT has been shown to detoxify copper in cell culture (16), it seems likely that MT-null mice will be more sensitive to copper toxicity.

MTs are also induced in many organs by inflammation. This induction can be mimicked by lipopolysaccharide and various cytokines such as interleukin 1, interleukin 6, and tumor necrosis factor α, all of which are released from activated macrophages in response to lipopolysaccharide (24). This suggests that MT induction might function to neutralize the excess reactive oxygen species generated by the granulocytes and monocytes. There is *in vitro* data indicating that MTs can scavenge hydroxyl radicals (25), and copper–MT, of either yeast or mammalian origin, can functionally substitute for superoxide dismutase in yeast (26). There are a variety of other xenobiotics that induce MT and many of them generate reactive oxygen (27). MT is induced in the lungs by elevated oxygen (28) and H_2O_2 can induce MT in some cultured cells (T. Dalton and G. Andrews, personal communication). Thus, there is mounting evidence that MT genes respond to oxygen and can help protect against its deleterious effects. Hence, it will be important to assess whether these MT-null mice are defective in their response to oxygen damage.

We anticipate that these MT-null mice and their transgenic counterparts that over-express MT-I by an order of magnitude (29) will provide valuable genetic tools for analysis of the physiological role of MT in response to a variety of environmental stresses.

We thank Phil Soriano, in whose lab the electroporation and selection of AB-1 cells was performed, Glenda Froelich for preparing the tissues for histology, and Denny Liggitt for his expert advice in evaluating the histological samples. We are grateful to Allen Bradley for AB-1 cells and Phil Soriano for the polNeo gene. We thank Dave Eaton and the members of the Palmiter lab for their careful reading of this manuscript. This work was supported in part by National Institutes of Health Grants HD-09172 and HD-23657. E.J.K. was supported by Public Health Service National Research Service Award T32 GM07270.

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