Metallothionein-I/II Double Knockout Mice Are Hypersensitive to Lead-Induced Kidney Carcinogenesis: Role of Inclusion Body Formation

Michael P. Waalkes,1 Jie Liu,1 Robert A. Goyer,1 and Bhalchandra A. Diwan2

1Inorganic Carcinogenesis Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute at the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina; and 2Basic Research Program, Science Applications International Corporation, National Cancer Institute at Frederick, Frederick, Maryland

ABSTRACT

Lead is an environmental nephrotoxicant and probable human carcinogen. Elucidating factors predisposing populations to lead toxicity is an important public health issue. Recently, we found that metallothionein-I/-II double knockout (metallothionein-null) mice that are unable to produce the major forms of metallothionein do not produce lead inclusion bodies, which are thought to mitigate lead toxicity and were sensitive to the subchronic toxic effects of lead exposure (10 weeks), showing modestly diminished renal function and nephromegaly compared with wild-type (WT) mice. It is unclear how this knockout might impact lead carcinogenesis. Thus, the effects of lead(II) acetate were tested in groups (n = 25) of male metallothionein-null and WT mice receiving drinking water with 0, 1,000, 2,000, or 4,000 parts per million lead for up to 104 weeks. Renal proliferative lesions (adenoma and cystic tubular atypical hyperplasia) were much more common and more severe in lead-exposed metallothionein-null mice than in WT mice. A metastatic renal cell carcinoma also occurred in a lead-treated metallothionein-null mouse, whereas none occurred in WT mice. Lead-induced renal proliferative lesions showed marked overexpression of cyclin D1, a common feature of human renal tumors. Renal lead-containing nuclear inclusion bodies were frequently observed in WT mice but did not form in metallothionein-null mice. Metallothionein was often found associated with the outer portion of these inclusion bodies. Thus, the metallothionein-null mice cannot form renal inclusion bodies, even after protracted lead exposure, and this increases the carcinogenic potential of lead. Poor production of metallothionein may predispose human populations to lead carcinogenicity.

INTRODUCTION

Lead is a naturally occurring, ubiquitous, environmental toxicant and potentially carcinogenic metal (1–7). Human lead exposure involves a substantial risk, particularly for the young (1–4, 7). The toxic effects of lead can manifest in various organs, and the kidney is clearly an important target (1–4, 7). Indeed, chronic renal lesions are considered one of the more insidious effects of the metal, in part because it may not be correlated with contemporaneous blood lead levels, which can be a poor indicator of past lead exposure (4). Lead induces chronic nephropathy and renal dysfunction often associated with hypertension and can occur in adults with a history of childhood lead poisoning (7). Children appear to be particularly sensitive to lead-induced chronic nephropathy and are considered a highly vulnerable risk group (8).

Inorganic lead compounds were recently reclassified as probably carcinogenic to humans (9, 10), and there is suggestive evidence linking renal cancer and lead exposure in humans (5, 6). Lead is an unequivocal nephrocarcinogen in rodents (1, 9, 10). In addition, lead commonly induces renal cystic tubular hyperplasia in rodents, considered a preneoplastic lesion leading to adenocarcinoma (11). There are gender- and age-related differences in sensitivity to lead-induced renal tumors in rodents (1, 12), although the basis of this sensitivity is uncertain. Additional research is clearly warranted to help additionally define the mechanisms and potential predisposing factors in renal lead toxicity and carcinogenicity.

A remarkable characteristic of lead poisoning is the production of a protein-lead complex, which appears in renal cells of poisoned humans or animals as inclusion bodies (1, 3, 4, 13). Lead-induced inclusion bodies are often nuclear, approximately spherical and consist of an electron-dense core with a peripheral fibrillar network (3, 4). Inclusion bodies first form in the cytoplasm and then migrate to the nucleus (3, 4, 13). Whether inclusion bodies are a permanent structure or decay with cessation of lead exposure is unknown. Similarly, the origin of the protein component of the inclusion bodies remains uncertain (3). Lead is highly concentrated in the inclusion bodies, and as much as half of the lead in the kidney may be found here (3, 4, 4). It is suspected that these complexes are protective in that they may render lead toxicologically inert, thereby blocking interactions with more sensitive cellular targets (3, 4, 13, 14). Thus, inclusion bodies may play a role in the intracellular partitioning and, possibly, toxicity of lead (4, 13–16). The role of inclusion bodies in lead carcinogenesis is undefined.

The metal-binding protein, metallothionein, is thought to play a role in the toxicology of a variety of metals (17–19) potentially including lead. Metallothionein can be a highly inducible protein, and metals are primary inducers (17, 19, 20). Lead avidly binds to metallothionein under ex vivo conditions (21), and lead appears to bind to metallothionein in human erythrocytes (22, 23). Metallothionein mitigates lead toxicity in cultured cells (13, 24), and the binding of lead to metallothionein reduces lead-induced inhibition of the enzyme δ-aminolevulinic acid dehydratase (25). Recently, we studied the role of metallothionein in the subchronic toxicity of lead, with metallothionein-null (metallothionein-I/-II double knockout) mice (13), which are unable to synthesize the two major forms of metallothionein, compared with wild-type (WT) mice, which produce metallothionein at normal levels. Ten weeks of exposure to lead in the drinking water in metallothionein-null mice did not produce overt nephropathy but did induce dose-related nephromegaly and modest reductions in renal function, effects absent in WT mice (13). This indicates that poor production of metallothionein may predispose mice to renal toxicity of lead (13). Unexpectedly, metallothionein-null mice showed a complete lack of lead-induced inclusion body formation (13). The sensitivity of metallothionein-null mice to lead was not based in altered biokinetics as metallothionein-null mice actually accumulated substantially less (~20%) renal lead than WT mice (13). Although this study supports a role for metallothionein in reducing the subchronic toxicity of lead (13), what role metallothionein might play in lead carcinogenesis is unclear, particularly in light of reduced renal accumulation of the metal in metallothionein-null mice. Thus, the present study was designed to investigate the role of metallothionein in chronic lead nephrotoxicity and carcinogenesis with metallothionein-null mice.

Received 6/23/04; revised 8/30/04; accepted 9/2/04.

Grant support: The National Cancer Institute and National Cancer Institute Contract NCI: CO-12400.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Michael P. Waalkes, Inorganic Carcinogenesis Section, National Cancer Institute at National Institute of Environmental Health Sciences, 111 Alexander Drive, Research Triangle Park, NC 27709. Phone: (919) 541-2328; Fax: (919) 541-3970; E-mail: waalkes@nihs.nih.gov.

©2004 American Association for Cancer Research.
Materials and Methods

Animals and Treatments. Animal care was provided in accordance with the United States Public Health Policy on the Care and Use of Animals as defined in the Guide to the Care and Use of Animals (NIH Publication 86-23). Homozygous metallothionein-II knockout mice (129-Mt1/H9262, M22/2MoLu) 129/SvPCy1 background; ref. 26) and corresponding WT mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The homozygous mutants were mated inter se to maintain the line. Mice were housed in a standard barrier facility, at a temperature of 68 to 72°F and with a relative humidity of 50 ± 5%, and a 12-hour light/dark cycle. Diet (NIH-31 Block Modified 6% fat;Ralston Purina, St. Louis, MO) and water were provided ad libitum. The NCI-Frederick animal program and facility, where the mice were housed, are accredited by the American Association for Accreditation of Laboratory Animal Care. At 8 weeks of age, male metallothionein-null and WT mice were randomly divided into four groups of 25 mice each. They were then provided acidified drinking water to which lead(II) acetate (Sigma Chemical Co., St. Louis, MO) was added to give a final lead concentration of 0 (control), 1,000, 2,000, or 4,000 parts per million. Individual body weights were recorded weekly until week 26 and then at monthly or bimonthly intervals. Clinical signs were checked daily, and mice were sacrificed when significant clinical signs developed or at 104 experimental weeks.

General Pathology. A complete necropsy was done on all of the moribund animals, animals found dead, or on mice at the study termination. The following tissues were taken: kidneys, testes, liver, lungs, adrenals, spleen, thymus, pituitary, and all of the grossly abnormal tissues. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with H&E for histologic analysis. Pathological assessments were done in a blind fashion.

Renal Pathology. Incidence of renal lesions were determined by histologic analysis of a longitudinal midline section of both kidneys from each mouse. Grossly appearing renal lesions were sectioned separately. The multiplicity of renal proliferative lesions (cystic hyperplasia or tumor) is the average number of lesions observed in a single midline section of both kidneys. To quantify average severity of lead-induced renal proliferative lesions, all of the renal lesions were classified into one of the following groups: absent (severity score = 0); tubular cysts lined by a single layer of abnormal (atypical) hyperplastic epithelium (mild hyperplasia; severity score = 1); tubular cysts lined by two or more layers of abnormal hyperplastic epithelium (moderate hyperplasia; severity score = 2); adenoma characterized by tubular cysts with abnormal hyperplastic cells filling the entire tubular lumen (severe hyperplasia; severity score = 3); or carcinoma (severity score = 4). For incidence data, mice with multiple lesions of different severity were grouped by their most advanced proliferative lesion.

Chronic nephropathy incidence and severity were also quantitated. Lead-induced chronic nephropathy was characterized by tubular degeneration, mineralization, and interstitial fibrosis. To quantify average severity of lead-induced nephropathic lesions, all of the lesions were classified into one of the following groups: absent (severity score = 0); mild (severity score = 1); moderate (severity score = 2); or severe (severity score = 3). Mild nephropathy is considered an aging lesion in these mice.

The average number of renal nuclear inclusion bodies was assessed in control and lead-treated metallothionein-null and WT mice killed at the end of the study. In 4 mice/group, 200 nuclei from random fields of a midline section of the inner cortex were blindly scored for the presence of inclusion bodies.

Immunohistochemistry. The presence of metallothionein in inclusion bodies of lead-treated WT mice was studied immunohistochemically. A mouse monoclonal antibody (Zymed Laboratories, Inc., San Francisco, CA) against a conserved epitope of the I and II isoforms was used. Paraffin block sections (4 μm) were incubated with biotinylated primary antibody (dilution 1:500), and then with streptavidin-peroxidase, followed by reaction with diaminobenzidine as the chromagen. The nuclear counterstain was hematoxylin.

RESULTS

Survival and Body Weights. Male metallothionein-null and WT mice were placed into groups (n = 25) and given drinking water containing lead at concentrations of 0 (control), 1,000, 2,000, or 4,000 parts per million lead. Mice were killed when moribund or after 104 weeks of treatment. Survival at the end of the study in WT mice was as follows: control, 11 of 25; 1,000 parts per million lead, 8 of 25; 2,000 parts per million lead, 14 of 25; and 4,000 parts per million lead, 5 of 25. Survival at the end of the study in metallothionein-null mice was as follows: control, 11 of 25; 1,000 parts per million lead, 11 of 25; 2,000 parts per million lead, 7 of 25; and 4,000 parts per million lead, 3 of 25. Survival was significantly reduced (P < 0.05) compared with phenotypic controls in both the metallothionein-null and WT mice at 4,000 parts per million lead. A significant reduction in survival also occurred in metallothionein-null mice given 2,000 parts per million lead compared with WT mice given the same dose of lead. A significant dose-related reduction in survival occurred in metallothionein-null mice but not in WT mice. It is likely that kidney cancers and other renal lesions (see below) contributed to the suppression of long-term survival in metallothionein-null mice.

There were no differences in body weight between control WT and control metallothionein-null mice at any point in the study. The 1,000 parts per million lead dose had no impact on body weight relative to control in either WT or metallothionein-null mice. Significant depressions in body weight did occur in mice treated with 2,000 parts per million or 4,000 parts per million lead. In WT mice, body weight depression occurred starting after 82 weeks of lead exposure. From that point on, body weights in WT mice were suppressed 7 to 9% compared with control at 2,000 parts per million lead 12 to 14% at 4,000 parts per million lead. Metallothionein-null mice showed significant body weight suppression after 54 weeks of lead exposure. From that point on, body weight in metallothionein-null mice was suppressed 7 to 9% compared with control at 2,000 parts per million lead and 14 to 18% at 4,000 parts per million lead. Thus, lead-induced body weight suppression occurred 28 weeks earlier in metallothionein-null mice than in WT mice, although the levels of suppression were similar.
Renal Pathology. All aspects of lead-induced renal pathology were much more pronounced in metallothionein-null mice compared with WT mice. Pathological lesions arising from chronic lead exposure in metallothionein-null mice included renal adenomas characterized by tubular cysts with atypical hyperplastic cells filling the entire tubular lumen. An aggressive renal cell carcinoma also occurred in one metallothionein-null mouse given 2,000 parts per million lead in the drinking water, which metastasized to both the lung and to the adrenal gland. This tumor is remarkable in light of the rarity of spontaneous renal carcinomas in mice. Lead-induced renal proliferative lesions included tubular cysts lined by a single layer of abnormal hyperplastic epithelium (defined as mild hyperplasias) and renal tubular cysts lined by two or more layers of abnormal hyperplastic epithelium but not filling tubular lumen (defined as moderate hyperplasia). Pictorial examples of these lead-induced renal proliferative lesions in mice have been published previously (12).

Quantitatively, lead-induced renal tumors and hyperplasias were commonly observed in metallothionein-null mice and infrequently seen in WT mice (Table 1). In metallothionein-null mice, the incidences of renal adenoma, moderate hyperplasia, and total proliferative lesions (combined mild hyperplasia, moderate hyperplasia, adenoma, and carcinoma) showed significant increases over phenotypic control after lead exposure and strong dose-related trends across the three doses of lead (Table 1). In all, the 75 lead-exposed metallothionein-null mice had 10 renal adenoma and one renal carcinoma. In contrast, in the 73 lead-treated WT mice, only a single adenoma occurred, and the incidence of total renal proliferative lesions was only modestly increased at the highest lead dose. Although the incidence of total renal proliferative lesions showed a clear dose-response relationship in both WT and metallothionein-null animals, the maximum incidence was nearly 3-fold higher in metallothionein-null mice (60%) compared with WT mice (21%). In WT mice, nearly 80% of renal proliferative lesions were mild hyperplasia. In contrast, in lead-treated metallothionein-null animals, 83% of total proliferative lesions were diagnosed as moderate hyperplasias or tumors.

Not only was the incidence of renal proliferative lesions elevated in lead-treated metallothionein-null mice, but the severity and multiplicity of these lesions was also dramatically increased compared with WT mice (Fig. 1). Average severity of renal proliferative lesions (see Materials and Methods) was significantly increased over phenotypic control at all three doses of lead in the metallothionein-null mice but never reached statistical significance in WT mice (Fig. 1, left). With regard to the multiplicity of renal proliferative lesions (proliferative lesions/mouse), metallothionein-null animals showed a marked, dose-
All stages of renal proliferative lesions stained intensely (dark brown) in cortical fields was 0.0 average number of inclusion bodies/200 nuclei from random renal thionein-null mice regardless of lead exposure. In WT mice, the incidence and severity of inclusion bodies was significantly increased compared with WT control and metallothionein-null mice with kidney lesions including representative cystic hyperplastic tubules, and renal cell adenomas, as well as the lead-induced renal cell carcinoma and its pulmonary metastasis were used to evaluate the localization and intensity of cyclin D1. Representations of moderate hyperplasia (left) and carcinoma (right) are shown (×400). All stages of renal proliferative lesions stained intensely (dark brown) for cyclin D1, with prominent nuclear localization.

When overexpressed, cyclin D1 is considered an oncogene and is associated with various cancers, including human renal cell carcinoma (27, 28). To see if similar molecular changes occurred during lead carcinogenesis in metallothionein-null mice, lead-induced renal proliferative lesions were assessed immunohistochemically for cyclin D1 (Fig. 2). All stages of renal proliferative lesions stained intensely for cyclin D1, with prominent nuclear localization. Examples of staining in moderate hyperplasia and carcinoma are shown. The renal carcinoma metastasis to the lung also showed intense cyclin D1 staining (data not shown). Very weak or no cyclin D1 staining was observed in the nuclei and cells of the surrounding normal renal tubular epithelium.

Mild chronic nephropathy, a common aging lesion in mice, occurred to a similar extent in controls and lead-treated mice regardless of phenotype. The incidence of more serious chronic nephropathy (moderate or severe) was high in lead-treated metallothionein-null mice with a clear relationship to lead dose (Table 2). Lead-induced chronic nephropathy included tubular degeneration, necrosis, mineralization, and interstitial fibrosis. The severity of lead-induced nephropathy was also greatly increased in metallothionein-null mice (Fig. 3). In lead-treated WT mice, the incidence and severity of chronic nephropathy was not significantly different from control.

**Renal Inclusion Bodies.** Lead-induced nuclear inclusion bodies of kidney cells are considered pathognomonic of chronic lead exposure and did not occur in control WT or control metallothionein-null mice. These bodies were common in the kidneys of WT mice exposed to lead but were totally absent in kidneys of lead-treated metallothionein-null mice regardless of lead exposure. In WT mice, the average number of inclusion bodies/200 nuclei from random renal cortical fields was 0.0 ± 0.0 (mean ± SEM) in control, 11 ± 0.6 at 1,000 parts per million lead, 15 ± 0.6 at 2,000 parts per million lead, and 19 ± 0.9 at 4,000 parts per million lead. The number of inclusion bodies was significantly increased compared with WT control and dose matched metallothionein-null mice at all of the lead doses. A significant dose-related increase to over 2 lesions/mouse after lead exposure (Fig. 1, right). In fact, some lead-treated metallothionein-null mice had as many as 8 proliferative lesions. Lead exposure did not substantially increase renal proliferative lesions multiplicity at any dose of lead in WT mice.

Because metallothionein-null mice were unable to form lead-containing nuclear inclusion bodies, to see if metallothionein was involved with these inclusions in lead-treated WT mice, samples of renal cortex were subjected to immunohistochemical analysis for metallothionein. Many, but not all, lead-induced inclusion bodies stained intensely for metallothionein in lead-exposed WT mice, and it appeared to be associated with the surface of the structure (Fig. 4). In other inclusion bodies metallothionein staining was less intense or absent. To quantify the presence of metallothionein in these inclusion bodies, the intensity of metallothionein staining was scored as strong, moderate-to-weak, or absent. The presence of metallothionein staining was scored as strong, moderate-to-weak, or absent in 100 inclusions each from kidney sections of four different lead-treated WT mice. This analysis indicated 16 ± 1.2% (mean ± SEM) of the inclusion bodies showed strong staining for metallothionein, 28.7 ± 1.8% showed moderate-to-weak staining, whereas 55.3 ± 1.3 did not show significant metallothionein staining.

**Table 2: Effect of oral lead exposure on the incidence of chronic nephropathy in MT-null and WT mice.**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Lead dosage</th>
<th>n</th>
<th>Moderate</th>
<th>Severe</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Control</td>
<td>25</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>WT</td>
<td>1,000 ppm</td>
<td>24</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>WT</td>
<td>2,000 ppm</td>
<td>25</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>WT</td>
<td>4,000 ppm</td>
<td>24</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>MT-null</td>
<td>Control</td>
<td>25</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>MT-null</td>
<td>1,000 ppm</td>
<td>25</td>
<td>5</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>MT-null</td>
<td>2,000 ppm</td>
<td>25</td>
<td>8*</td>
<td>3</td>
<td>11*†</td>
</tr>
<tr>
<td>MT-null</td>
<td>4,000 ppm</td>
<td>25</td>
<td>10*</td>
<td>8*†</td>
<td>18*†</td>
</tr>
</tbody>
</table>

Trend $P = 0.0051$ $<0.0001$§

NOTE. Data expressed as number of mice bearing the specified grade of lesion. Total incidence includes only moderate to severe nephropathy, as mild chronic nephropathy was considered a common aging lesion.

Abbreviations: MT, metallothionein; ppm, parts per million.

* Significantly different ($P < 0.05$) compared to phenotypic control.
† Significantly different ($P < 0.05$) compared to dosage matched WT mice.
§ A significant trend ($P < 0.05$) across all doses within a phenotype.
Lead is considered one of the most hazardous contaminants of the human environment. Genetic susceptibility is a critical contributing factor to the widely different toxic responses in individuals exposed to similar levels of environmental toxicants potentially including lead (29, 30). The present work unequivocally demonstrates that the inability to express the major isoforms of the metallothionein gene created a profoundly heightened sensitivity to the nephrotoxic effects of lead in mice. This exacerbated toxic response includes nephrocarcinogenicity, as evidenced by a remarkable increase in incidence, severity and multiplicity of renal proliferative lesions, including tumors, in metallothionein-null mice. Indeed, the renal cell carcinoma observed in a lead-treated metallothionein-null mouse is remarkable because of the rarity of spontaneous renal carcinomas in mice. The observation of marked overexpression of cyclin D1 in lead-induced renal tumors and other proliferative lesions is consistent with its overexpression in human renal carcinoma (27, 28). The incidence and severity of chronic lead nephropathy was also greatly increased in metallothionein-null mice but did not occur to a significant extent in WT mice. In addition, the ability to synthesize metallothionein appears mechanistically linked to formation of lead-induced inclusion bodies, which, in turn, appear to be critical in the adaptive response of target renal cells to the chronic toxic effects of inorganic lead. The actual association of metallothionein with a subset of lead-induced nuclear inclusion bodies in WT mice strongly points to a significant role for this protein in their formation. Given the concern that there may be no lower threshold for the adverse effects of lead in exposed human populations (31, 32), any factor that predisposes individuals to

**Nonrenal Pathology.** Differences in hepatocellular tumor incidence occurred with WT and metallothionein-null mice (Table 3). Control metallothionein-null mice had a higher spontaneous rate of total hepatocellular tumors (52%) compared with WT control mice (24%). Furthermore, no hepatocellular carcinomas occurred in the 98 total WT mice, whereas 6 hepatocellular carcinoma occurred in the 100 metallothionein-null mice (P = 0.015). Unexpectedly, lead suppressed hepatocellular tumor formation in both the metallothionein-null and WT mice.

Lung tumors were common and not altered by lead exposure. Lung tumors in WT mice included the following: control, 8 adenoma and 7 carcinoma; 1,000 parts per million lead, 5 adenoma and 8 carcinoma; 2,000 parts per million lead, 8 adenoma and 5 carcinoma; and 4,000 parts per million lead, 6 adenoma and 8 carcinoma. Lung tumors in metallothionein-null mice included the following: control, 7 adenoma and 8 carcinoma; 1,000 parts per million lead, 8 adenoma and 4 carcinoma; 2,000 parts per million lead, 5 adenoma and 7 carcinoma; and 4,000 parts per million lead, 5 adenoma and 4 carcinoma.

Other spontaneous tumors not altered by lead exposure in metallothionein-null mice (n = 100) included the following: 6 lymphoma, 3 testicular teratoma, 3 hemangiosarcoma, 3 hemangioma, a Harderian gland adenoma, a pituitary adenoma, a lymphosarcoma, a cholangioma, and an adrenal cortical adenoma. Spontaneous tumors that were not associated with lead exposure in WT mice (n = 98) included the following: 3 testicular teratoma, 2 hemangiom, 3 hemangiosarcoma, 2 Harderian gland carcinoma, 2 Harderian gland adenoma, 2 lymphoma, a pituitary adenoma, a lung mesothelioma, a pancreatic islet cell adenocarcinoma, and a forestomach squamous cell carcinoma.

**DISCUSSION**

Lead is considered one of the most hazardous contaminants of the human environment. Genetic susceptibility is a critical contributing factor to the widely different toxic responses in individuals exposed to similar levels of environmental toxicants potentially including lead (29, 30). The present work unequivocally demonstrates that the inability to express the major isoforms of the metallothionein gene created a profoundly heightened sensitivity to the nephrotoxic effects of lead in mice. This exacerbated toxic response includes nephrocarcinogenicity, as evidenced by a remarkable increase in incidence, severity and multiplicity of renal proliferative lesions, including tumors, in metallothionein-null mice. Indeed, the renal cell carcinoma observed in a lead-treated metallothionein-null mouse is remarkable because of the rarity of spontaneous renal carcinomas in mice. The observation of marked overexpression of cyclin D1 in lead-induced renal tumors and other proliferative lesions is consistent with its overexpression in human renal carcinoma (27, 28). The incidence and severity of chronic lead nephropathy was also greatly increased in metallothionein-null mice but did not occur to a significant extent in WT mice. In addition, the ability to synthesize metallothionein appears mechanistically linked to formation of lead-induced inclusion bodies, which, in turn, appear to be critical in the adaptive response of target renal cells to the chronic toxic effects of inorganic lead. The actual association of metallothionein with a subset of lead-induced nuclear inclusion bodies in WT mice strongly points to a significant role for this protein in their formation. Given the concern that there may be no lower threshold for the adverse effects of lead in exposed human populations (31, 32), any factor that predisposes individuals to

![Fig. 3. Severity of lead-induced chronic nephropathy in WT and metallothionein-null mice exposed to lead in the drinking water. Severity was blindly scored as absent (score = 0), mild (1), moderate (2), or severe (3) [see Materials and Methods]. Bars, ±SEM (n = 24 to 25). * Significant difference from phenotypic control; †, significant difference from dosage matched WT mice. (MT, metallothionein; ppm, parts per million).](image)

![Fig. 4. Immunohistochemical analysis of metallothionein staining intensity and localization in lead-induced nuclear inclusion bodies from the kidneys of WT mice. A mouse monoclonal antibody against a conserved epitope of metallothionein-I and metallothionein-II isoforms was used. Representative section including inclusion bodies showing intense surface staining (dark brown; ×400).](image)

![Table 3 Effect of oral lead exposure on the incidence of hepatic tumors in MT-null and WT mice](image)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Lead dosage</th>
<th>n</th>
<th>Adenoma</th>
<th>Carcinoma</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Control</td>
<td>25</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>WT 1,000 ppm</td>
<td>24</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>WT 2,000 ppm</td>
<td>25</td>
<td>0*</td>
<td>0</td>
<td>0</td>
<td>0*</td>
</tr>
<tr>
<td>WT 4,000 ppm</td>
<td>24</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Trend P</td>
<td>0.035‡</td>
<td></td>
<td></td>
<td></td>
<td>0.035‡</td>
</tr>
<tr>
<td>MT-null Control</td>
<td>25</td>
<td>10</td>
<td>3</td>
<td>13‡</td>
<td></td>
</tr>
<tr>
<td>MT-null 1,000 ppm</td>
<td>25</td>
<td>8</td>
<td>1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>MT-null 2,000 ppm</td>
<td>25</td>
<td>2*</td>
<td>2</td>
<td>4*</td>
<td></td>
</tr>
<tr>
<td>MT-null 4,000 ppm</td>
<td>25</td>
<td>1*</td>
<td>0</td>
<td>1*</td>
<td></td>
</tr>
<tr>
<td>Trend P</td>
<td>0.0003‡</td>
<td></td>
<td>0.13</td>
<td>&lt;0.0001‡</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** Data expressed as number of mice bearing the specified grade of liver tumor. Data concerns only mice available for pathological analysis.

In cases of multiple tumors in the same tissue, the animal is assigned to the appropriate group based on its most advanced lesion. Total tumor incidence is defined as those mice bearing at least one benign or malignant hepatocellular tumor.

Abbreviations: MT, metallothionein; ppm, parts per million.

* Significantly different (P < 0.05) compared to phenotypic control.

† Significantly different (P < 0.05) compared to dosage matched WT mice.

‡ A significant trend (P < 0.05) across all doses within a phenotype.
lead toxicity takes on great importance. In this regard, control of metallothionein expression follows a complex, incompletely defined pattern dictated by factors like tissue, cell type, metal exposure, ontogeny, gender, and physiologic or pathological status, to name a few (17, 20). In humans, interindividual metallothionein expression can vary greatly even in the same cell type (33, 34) for reasons that are not completely understood. For instance, in human blood lymphocytes from 16 individuals, the expression of metallothionein after in vitro exposure to a metal inducer showed a 35-fold difference from the lowest to the highest responder (33), whereas in autopsy samples of kidney from Japanese adults, there is a distinct subgroup that poorly expresses renal metallothionein (35). The results of the present work indicate that such variability in expression might well impact renal toxicity and carcinogenicity in humans after lead exposure.

It appears that a subpopulation of lead-exposed workers can exhibit lead intoxication at relatively low blood lead levels (36), and lead toxicity in humans is known to be dictated by individual susceptibility (22). In this regard, a comparative study of lead speciation in erythrocytes in two lead-exposed workers provides evidence of a specific role for metallothionein in the sensitivity to lead toxicity (22). Although both workers were heavily exposed and had similar blood lead levels, one displayed clear symptoms of lead toxicity, whereas the other was totally asymptomatic (22). In the asymptomatic worker, ~70% of erythrocytic lead was associated with a metallothionein-like protein compared with only 20% in the lead-intoxicated individual (22). Additional study of this lead-binding protein showed biochemical characteristics consistent with metallocionine (22, 23). The limited number of subjects in this series of studies (22, 23) dictates caution when interpreting these findings, and there is no clear evidence that blood-borne metallocionine levels correlate with levels in other tissues like the kidney. However, the remarkable interindividual variability in human metallocionine expression (33, 34) together with the clear correlation between poor expression of metallocionine and lead-induced renal toxicity and carcinogenesis in mice (present study) indicate that this metal-binding protein could be an important factor in human susceptibility to lead. Additional research on metallocionine as a component of human sensitivity to lead toxicity is clearly warranted.

Although malignant renal tumors were uncommon in the present study, there was a marked increase in the incidence of renal adenoma and cystic hyperplasia, which are considered precursor lesions to renal cell carcinoma, in lead-treated metallocionine-null mice compared to WT mice. Furthermore, both the multiplicity and severity of renal proliferative lesions were markedly increased in lead-treated metallocionine-null animals. Lead-induced renal cancers in rodents are often found against a background of preneoplastic renal epithelial cell hyperplasia (1, 12, 37), as was observed in the present study, fortifying the concept that they are indeed premalignant lesions. In this regard, poor metallocionine expression has been associated with renal cell carcinoma in several studies (38–40). For instance, in a recent gene expression profiling survey of human renal cell carcinomas, coordinate down-regulation of all of the metallocionine forms was a common change in gene expression (38). Others have detected a reduction in metallocionine expression in human renal carcinomas in comparison to non-neoplastic tissue from the same patients (39, 40). Whether metallocionine expression is a cause or consequence of renal cancer cannot be defined by these studies. However, given the remarkably heightened sensitivity to lead-induced, proliferative lesions that occurred in mice poorly expressing metallocionine throughout their life and observed reductions in metallocionine expression in human renal cell carcinomas (38–40), it would be of interest to determine whether poor metallocionine expression played a general role in sensitivity to renal carcinogenesis. Indeed, based on diminished mutation rates in metallocionine-overexpressing cells, and because there is variability in human metallocionine expression, it has been proposed that low metallocionine expression might be a general risk factor for cancer (41).

The inability to produce inclusion bodies on chronic exposure to lead was associated with the high frequency and severity of renal proliferative lesions and chronic nephropathy in metallocionine-null mice. In WT mice, which displayed much more limited renal pathology after chronic lead exposure, inclusion bodies were common in kidney cells. Additionally, a substantial number (16%) of lead-induced inclusion bodies stained intensely for the presence of metallocionine, and this metallocionine appeared to be associated with the outer surface of the inclusions. This might indicate some function for metallocionine in initial lead transport or could reflect aberrant essential metal trafficking induced by lead. In our initial screening of the immunohistochemical localization of metallocionine in inclusion bodies (13), we found no clear association between metallocionine and the inclusions. This is perhaps because of the limited number of sections investigated in our initial study (13) or reflects a difference in the length of lead exposure, which in the present study was up to 2 years but in our prior work was only for 10 weeks (13). In any event, it is clear from the present work and our earlier subchronic study (13) that metallocionine plays a key role in production of lead-induced inclusion body formation, although its exact role remains to be established. It has been proposed that inclusion bodies are a cellular adaptation mechanism whereby lead is sequestered in a relatively inert and, therefore, nontoxic form (3, 4, 13, 14). The results of the present study provide unequivocal evidence that the inability to form inclusion bodies greatly exaggerates the chronic toxicity of lead to the kidney. We are now directing research to determine whether metallocionine facilitates the formation or is actually part of the lead-induced inclusion bodies.

Interestingly, a recent test of acute lethality of various metals in metallocionine-null and WT mice with an escalating dosage scheme found that metallocionine provided no protection against the acute lethal effects of injected lead (19). Thus, it appears that although metallocionine reduces the subchronic and chronic toxic effects of lead, it does not prevent events associated with high doses and early lethality (19). In this regard, in vitro studies have shown inclusion bodies take at least 24 hours after lead exposure to be formed in kidney cells (42). These data indicate that inclusion bodies are an adaptive response that is not normally operating and only becomes functional some time after the initial exposure to lead. As such, it would appear that different factors dictate sensitivity to the acute versus the chronic toxic effects of lead.

Control metallocionine-null mice had a higher incidence of spontaneous hepatocellular tumors than control WT mice. This included several cases of hepatocellular carcinoma in metallocionine-null mice, whereas only benign tumors occurred in WT mice. The increase in liver cancers in control metallocionine-null mice is interesting in light of the report that metal-induced overexpression of hepatic metallocionine greatly reduces spontaneous liver tumors in C57BL/6 mice (43). Furthermore, metallocionine-transgenic mice that constitutively overexpress metallocionine-I at levels up to 20× WT control, also show a marked decrease in spontaneous tumor formation (44). Because increased cellular metallocionine is correlated with reduced spontaneous mutations, it is thought that low metallocionine expression could be a general risk factor for tumor development (41). The present findings provide additional evidence that the presence of metallocionine may reduce spontaneous carcinogenesis. In addition, lead exposure suppressed hepatocellular tumor formation in both metallocionine-null and WT mice. Other metals, like cadmium (45),...
can reduce liver tumor formation in rodents, although the mechanism for this suppression is not known.

In summary, metallothionein-null mice are unable to form renal inclusion bodies in response to chronic lead exposure and are remarkably susceptible to development of lead-induced neoplastic, preneoplastic, and chronic toxic kidney lesions. Although the exact role of lead-induced inclusion body formation is not known, our results strongly suggest that the inability to produce these lead-containing bodies renders mice hypersensitive to the carcinogenic and chronic toxic effects of this metal. Efforts to define the role of metallothionein in the sensitivity of human populations to lead intoxication should be a research priority.

ACKNOWLEDGMENTS

The authors wish to thank Dan Logsdon for excellent technical assistance and Dr. Jerrold M. Ward for assistance with pathological analysis and photomicrographs.

REFERENCES

Metallothionein-I/II Double Knockout Mice Are Hypersensitive to Lead-Induced Kidney Carcinogenesis: Role of Inclusion Body Formation

Michael P. Waalkes, Jie Liu, Robert A. Goyer, et al.

Cancer Res 2004;64:7766-7772.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/21/7766

Cited articles
This article cites 38 articles, 8 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/21/7766.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/64/21/7766.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.