Correlation between Cadmium-induced Pulmonary Carcinogenicity, Metallothionein Expression, and Inflammatory Processes: A Species Comparison

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There is sufficient evidence for pulmonary carcinogenicity of inhaled Cd compounds in rats whereas no such evidence was found in mice and hamsters; the evidence in humans has been termed limited, indicating significant species differences in pulmonary response to inhaled Cd. We hypothesized that expression of metallothionein (MT) protein in the lung after inhalation of Cd differs between species thereby providing different degrees of sequestration of Cd and protection from its effects. Rats and mice were exposed to 100 μg CdCl₂ aerosols/m³ for 4 weeks, and the presence of MT was determined in lung and free lung cell homogenates as well as by immunocytochemistry in lung sections up to 28 days postexposure. In addition, pulmonary inflammatory, and cell proliferative responses were determined. Cd exposure significantly increased MT in homogenates of total lung in both species; however, no significant increase of MT in rat lung tissue after removal of free lung cells by lavage was found whereas MT was still significantly increased in lavaged mouse lung tissue throughout the postexposure time. In contrast, exposed rats showed significant increases in MT in the lavageable lung cells and mice did not. Histochemical analysis of lung sections revealed that mainly the epithelial cells of the bronchi, bronchioi, and alveoli of Cd-exposed mice expressed MT. Mice also exhibited a marked and sustained pulmonary inflammatory and cell proliferative response upon CdCl₂ exposure which was not observed in rats. The retained Cd dose per gram lung was about 2-fold greater in mice, which is consistent with a greater deposition efficiency of inhaled Cd-aerosols in mice. The pulmonary Cd retention halftime was about twice as long in mice than in rats. The greater MT induction may offer an explanation for the resistance of mice toward the pulmonary carcinogenic effect of inhaled Cd despite a greater pulmonary inflammatory and cell proliferative effect and a greater retention halftime of lung Cd in this species. — Environ Health Perspect 102(Suppl 3):257–263 (1994).

Key words: cadmium, metallothionein, carcinogenesis, lung, inflammation, cell proliferation, rat, mouse

Introduction

CdCl₂ aerosol inhalation by rats over an 18-month period resulted in a significant dose-dependent induction of lung tumors at concentrations ranging from 12.5 μg/m³ to 50 μg/m³ (1). The highest concentration resulted in lung tumors in over 70% of the rats. This was a potentially alarming finding in view of the fact that occupational exposure limits at the time of the report were generally at 50 μg/m³, and even as high as 100 to 200 μg/m³ in the United States. Subsequent chronic exposure studies in rats with several different Cd compounds (i.e., CdSO₄, CdO-dust, CdO-fume, CdS and CdCl₂) corroborated the earlier findings by demonstrating a similar pulmonary carcinogenic response from these inhaled Cd compounds (2). In contrast to these unequivocal findings in rats, Heinrich et al. (3) did not observe a significant pulmonary carcinogenic response in chronic inhalation studies in mice and hamsters using the same Cd compounds and exposure concentrations. The evidence that Cd is a pulmonary carcinogen in humans has been termed limited by International Agency for Research on Cancer (IARC) (4) based on a number of epidemiologic studies that did not uniformly show a significant correlation between Cd inhalation exposure and lung cancer; this issue is still controversial (5,6).

The results of experimental animal and epidemiologic studies suggest that the pulmonary tumorigenic potency of inhaled Cd compounds is quite different in different mammalian species. An important question relates to the most appropriate animal model for extrapolation to humans: Do rats, or mice and hamsters make better models to study mechanisms of pulmonary carcinogenesis relevant for humans? Another question arising from the animal data concerns the design of new studies to investigate mechanisms that may explain the observed species differences in pulmonary Cd carcinogenesis. A basic knowledge about the mechanisms involved in various species for the different responses (for example in rats vs. mice) would also contribute greatly to our understanding of the human disease process.

The studies reported in this paper were an attempt to compare the pulmonary responses in rats and mice after subchronic inhalation exposure to CdCl₂ aerosols. Our hypotheses were that rats and mice respond differently to the same inhaled Cd concentration with respect to pulmonary inflammatory and epithelial cell proliferative responses (mice < rats) and inducibility of metallothionein (MT) (mice > rats). These
hypotheses were based on findings from particle inhalation studies that showed that mice responded with a lower pulmonary inflammation and cell proliferation than rats when chronically exposed to the same particle concentration [e.g., talc particles, NTP (7)] and that MT will bind and sequester Cd, thereby diminishing its potential for cell and DNA damage (8). The latter mechanism is also supported by findings in the chronic inhalation studies with Cd in rats (2) which showed that co-inhalation of rats to CdO + ZnO aerosols could prevent the induction of lung tumors and by our earlier results showing that inhalation of ZnO is a much stronger inducer of MT in the rat lung than CdO (9).

**Methods**

Forty-eight male Fischer-344 rats, body-weight 191 ± 12 g, and 72 male Balb/C mice, body-weight 18.6 ± 0.5 g, were exposed in whole-body exposure chambers to nominally 100 μg Cd/m³ as CdCl₂ aerosols for 6 hr/day, 5 days/week for 4 weeks. The CdCl₂ aerosols were generated with an ultrasonic nebulizer (Heyer Co, Bad Ems, Germany). The particle size as measured by an 8-stage Mercer impactor (Intox Albuquerque, NM) was 0.38 μm (mass median aerodynamic diameter) with a geometric standard deviation of 1.41. Control rats and mice were sham-exposed to filtered air. Groups of rats were sacrificed at 1 day, 7 days, and 28 days after termination of exposure. The Cd dose in the lung, liver, and kidney was determined at 1 day after exposure following tissue digestion via Cd analysis by atomic emission spectrometry. At all sacrifice timepoints, extensive bronchoalveolar lavages (BAL) were performed by lavaging the excised lungs ten times with a volume of 5 ml (rats) or 1 ml (mice) of saline. Cell differential, protein content, and lactate dehydrogenase (LDH) and β-glucuronidase in the lung lavage were determined as indicators of an inflammatory response. The pulmonary circulation was saline-perfused via the right heart. The right lung lobe was flash-frozen in liquid nitrogen for measurement of MT protein by the silver-heme method (10). The left lung lobes were fixed in 10% formalin and prepared for histology and for immunohistochemistry of MT protein using polyclonal antibodies raised in rabbits (11). After centrifugation of the lavaged cells at 400 g for 10 min, the cell pellet and supernatant were also analyzed for MT protein.

Cellular proliferative responses were determined by immunohistochemical methods using 5-bromo-2’-deoxyuridine (BrDU) subcutaneously administered via osmotic minipumps and by specific staining for proliferating cell nuclear antigen (PCNA). For the BrDU administration, Alzet osmotic minipumps (Alzet Corp., Palo Alto, CA), model 2ML1 for rats and model 2001 for mice, were used. The delivery rates of these pumps are 10 μl/hr and 1.0 μl/hr, respectively. After subcutaneous implantation, the pumps remained for 4.5 days before the animals were sacrificed for further tissue fixation and preparation. BrDU concentration in the Alzet osmotic minipumps was 20 mg/ml. Tissues were fixed in Bouin’s fixative by intratracheal fixative instillation and immersion. Specific BrDU and PCNA staining was performed by Veritas Laboratories (Burlington, NC) by using general methodologies described by Goldsworthy (12) and van Dierendonck (13). For PCNA staining, methods were used as described by Hall (14), Greenwell (15), and Foley (16). The use of both markers for cell proliferation became necessary since at the earlier sacrifice timepoints (1 and 7 days postexposure) the subcutaneous BrDU administration had been unsatisfactory. A comparison of the exogenous marker, BrDU, with the endogenous marker, PCNA, in our previous studies (inhaled solid particles) had shown an excellent agreement with respect to labeling indices (unpublished). Indeed, in our present studies the 28 day sacrifice timepoint again showed an excellent concordance of both labeling techniques (Results). BrDU- or PCNA-stained slides were examined at ×400 using an Olympus AH2 microscope fitted with a Spectra Services data analysis system. Three replicate fields, each consisting of 20,000 μm² by 5 μm thick were examined. Results are expressed as number of cells per 10⁴ mm².

**Results**

At the end of the 4-week exposure, the total Cd in the lungs of rats was 7.4 ± 0.7 μg and in mice 2.2 ± 0.4 μg (Table 1). Normalized for lung weight, the respective lung Cd concentrations were 7.2 ± 0.4 μg/g lung (rats) and 15.0 ± 0.5 μg/g lung (mice). Thus, mice retained twice as much of the inhaled Cd per gram lung than rats. There was also a difference with respect to the cellular distribution of retained Cd within the lung. In rats, 12% was retained in the lavageable cells (mainly alveolar macrophages) and 87% was retained in the lavaged lung tissue. In contrast, mice retained only 2% in the lavageable cells and 97% was retained in their lung tissues. In both species, about 1% of the total lung

| Table 1. Pulmonary Cd content after a 4-week exposure to CdCl₂ aerosol, 100 μg/m³ |
|---------------------------------|---------------------------------|---------------------------------|
| Rats                            | Mice                            |
| Follow-up                        | Follow-up                        | Follow-up                        | Follow-up                        |
| μg Cd/lung                       | μg Cd/g lung                     | μg Cd/lung                       | μg Cd/g lung                     |
| Control                          | Exposed                         | Control                          | Exposed                         |
| 0.008 ± 0.02                     | 7.39 ± 0.69                     | 0.028 ± 0.02                     | 7.22 ± 0.41                     |
| 0.028 ± 0.02                     | 2.20 ± 0.40                     | 0.182 ± 0.13                     | 15.00 ± 0.50                     |

n = 4.

\[ \text{pg} \pm \text{SEM} \]

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![Figure 1](https://via.placeholder.com/150)

**Figure 1.** Relative PMN numbers in BAL of rats and mice at different timepoints after a 4-week exposure to CdCl₂ (100 μg/m³, n = 4 per group). Rats = open bars; mice = hatched bars; control = 100%.

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which, however, did not reach a level of statistical significance (Table 2). The cellular inflammatory response in the mouse lungs was mirrored in a significant increase in the lysosomal and cytoplasmic enzymes \( \beta \)-glucuronidase and LDH during the post-exposure period but they did not change in rats (Table 2). Viability of the lavaged cells as determined by trypan blue exclusion was not significantly changed in both species, and ranged from 96 to 97% and from 92 to 95% in control and exposed rats and mice, respectively.

Cellular proliferative responses also showed significant differences between the two species: The number of PCNA-labeled cells of the alveolar epithelium was markedly increased in the \( \text{CdCl}_2 \)-exposed mice at 1 and 7 days postexposure compared to control mice. \( \text{CdCl}_2 \)-exposed rats did not show such dramatic increase in labeled epithelial cells. The pronounced cell proliferative response in \( \text{CdCl}_2 \)-exposed mouse lungs was still present at 28 days postexposure as demonstrated in Figure 2 by the increased BrDU label. The proliferative response appears to parallel the persistent alveolar inflammatory response observed by BAL analysis. In contrast, the exposed rats do not show a significant increase of BrDU-labeled cells (Figure 2). Quantitation of these observations is depicted in Figure 3, which shows significant increase in cell labeling of macrophages and epithelial cells in exposed mice but not in rats. Lung sections of these animals stained for PCNA showed the same qualitative differences between exposed and control rats and mice except that not as many cells appeared to show the label; that is probably due to the administration of BrDU over a prolonged period of 4.5 days (Methods).

Pulmonary baseline concentrations of MT were 12.34 ± 4.3 \( \mu \)g/g lung in control rats and 25.29 ± 5.99 \( \mu \)g/g lung in control
mice (Table 3). On day 1 after the 4-week exposure, these concentrations increased to 21.71 µg/g lung and 46.49 µg/g lung in rats and mice, respectively. Total MT levels in the lungs were significantly increased in both rats and mice at all time points after CdCl₂ exposure relative to the control animals (Table 4). However, there were marked differences between rats and mice when the MT levels of the lavaged cells and those in the extensively lavaged lungs were compared separately (Figure 4). In the lavaged lungs of rats, MT levels changed only slightly and showed a significant increase (∼1.4-fold) only at day 7 after exposure. In mice, on the other hand, a 3-fold increase in lung MT was observed throughout the postexposure period (Figure 4).

Quite in contrast to the MT increases in the lavaged lung tissue, the lavaged cells from the lungs of mice did not show any change in MT levels after CdCl₂ exposure, whereas the rats showed a large increase in MT content per lavaged cell, up to 6.5-fold, which persisted throughout the postexposure period (Figure 4). There was also a significant difference in baseline levels of MT in the lavaged cells (∼99% alveolar macrophages) between control rats and mice. The control mice had an almost 20-fold greater MT concentration per lavaged cell than control rats (Table 3). However, since control mice had about 20-fold fewer lavageable cells than rats, the absolute MT content in the free lung cells of both species was not significantly different. Likewise, baseline MT concentration per g control lung after removal of the free lavageable cells through extensive lung lavage was not significantly different between control rats and mice (Table 3).

The finding of significant differences in pulmonary MT induction between rats and mice after CdCl₂ exposure was corroborated by results from immunohistochemical MT staining: There was intense staining for MT in the epithelial lining cells of the conducting airways and alveolar region in the exposed mouse lungs, while there was only diffuse staining for MT in these regions in lungs of the exposed rats (Figure 5).

**Discussion**

Our hypothesis that Cd inhalation exposure leads to a greater induction of MT in the lungs of mice than in rats seems to be confirmed by the results of the present study with regard to the lung tissue. After removal of the lavageable free lung cells by extensive bronchoalveolar lavage, the lavaged lungs of mice showed up to 3-fold greater MT levels following a 4-week exposure to CdCl₂ aerosols compared to controls, whereas the lungs of exposed rats did not show this significant increase (Figure 4). However, quite the opposite results were observed with respect to MT induction in the lavaged free cells. After the 4-week CdCl₂ aerosol exposure, a marked increase in MT per lavaged cell was found in rats but not in the exposed mice (Figure 4). This species-specific differential increase in pulmonary MT content was not seen upon analysis of the total lung, i.e., lavaged lung tissue plus lavaged free lung cells. In this case, both rats and mice showed a significant increase in lung MT (Table 4), but it was more prominent in mice than in rats.

The lavaged cells consisted almost exclusively of alveolar macrophages in exposed and control rats and in control mice, whereas exposed mice had a significant fraction of PMN among the lavaged cell population. However, since MT increase in lavaged cells was only observed in rats, it is obvious that this increase occurred in the alveolar macrophages; whether this is due to increased MT induction in alveolar macrophages or to a possible uptake of alveolar macrophage extracellular MT induced by other cells in the alveolar region was not directly investigated. However, since no significant MT increase was found in the lavaged lung tissues (Figures 4, 5) of rats and since no MT was detectable in the BAL supernatant, we believe that an increased expression of MT in alveolar macrophages had occurred. This is also corroborated by results from Hart and Garvey (17) who showed a significant increase of MT levels in rat alveolar macrophages after Cd inhalation exposure. Previously they had also reported a significant increase in lung MT in rats in a dose-related response to inhaled Cd acetate in rats (18), which is in line with the overall increase of MT we found in rat lungs without lavage in this study. The much greater increases in lung MT observed by Hart et al. (18) in rat lungs could be explained by much greater Cd lung burdens in their study. In our present study, Cd levels in rat lungs were 7.4 µg/lung, whereas in the study by Hart et al. (18) pulmonary Cd levels ranged from a low of 10 µg/lung to a high of 43 µg/lung.

The significant increase of lung MT (lavaged lung) in mice but not in rats after exposure of both species for the same

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**Table 4**: Total MT in lung after a 4-week exposure to CdCl₂ aerosol, 100 µg/m³.

| Days after Exposure | Control (Rats) | Exposed (Rats) | Control (Mice) | Exposed (Mice) |
|---------------------|---------------|---------------|---------------|---------------|
|                     | µg MT/lung    | µg MT/lung    | µg MT/lung    | µg MT/lung    |
| 1                   | 11.79 ± 4.11  | 22.24 ± 1.66* | 3.32 ± 0.83   | 7.25 ± 2.21*  |
| 7                   | 13.32 ± 2.42  | 26.88 ± 4.19* | 3.31 ± 0.91   | 7.88 ± 1.61*  |
| 28                  | 27.14 ± 5.01  | 36.64 ± 2.76* | 7.73 ± 2.33   | 15.06 ± 2.16* |

*Significantly different from control, p < 0.05 (t-test)
length of time and to the same concentration of CdCl₂ aerosols may provide a greater protection against a potential pulmonary carcinogenic effect of Cd for mice. Such an effect of inhaled Cd compounds was only observed in rats (2) but not in mice or hamsters (3). The tumors observed in those rat studies originated from epithelial cells in the bronchiolar/alveolar region of the lung, and our present studies show a significant induction of MT after CdCl₂ inhalation in epithelial cells of both the conducting airways and the alveolar region of the mouse (Figure 5). It is, therefore, conceivable that retention of Cd in these cells via sequestration in MT could prevent any potential genotoxic interaction of Cd with these cells; the exact mechanism of such interaction still needs to be investigated. Such a protective effect of MT against the carcinogenic effects of Cd has also been suggested for other organs (8,19).

An interesting observation in our studies is that baseline levels of lung tissue MT (per gram lavaged lung, Table 3) were not significantly different between control rats and mice. On the other hand, total baseline MT concentrations per gram of lung (including lavageable lung cells) were significantly greater in mice due to a higher baseline level of MT in mouse alveolar macrophages (Table 3). Such species differences in MT levels in the unexposed control organ as well as a differential induction upon exposure to Cd have to be considered when evaluating effects on MT expression.

Based on results of a greater inflammatory and cell proliferative response in the lungs of mice as compared to rats following exposure to particles (7) we had hypothesized that we would also see a similar species difference after CdCl₂ inhalation. In the National Toxicology Program (NTP) (7) study with rats and in other particle studies (20), only rats showed a pulmonary carcinogenic response whereas mice did not, consistent with the greater inflammatory and cell proliferative response in rats compared to mice. However, our present results after CdCl₂ exposure of both species are not consistent with our hypothesis; quite in contrast, mice responded with a much greater and persistent pulmonary inflammation than rats after the same exposure to CdCl₂ aerosols, and cellular proliferative responses were also markedly greater in the CdCl₂-exposed mouse lungs compared to the rats. This unexpected result is in contrast to the idea that cell proliferative effects might be correlated with an increased risk of tumor incidences (21) since the opposite has been found after chronic Cd inhalation. Only rats but not mice responded with a significant and large increase in lung tumors.

The resistance of mice against Cd-induced lung tumors in spite of the significant responses in inflammation and cell proliferation should be viewed in the context of increased MT expression. It appears that mice are more sensitive than rats to a pulmonary inflammatory response of inhaled cadmium. Proinflammatory cytokines such as TNF-α and IL-1, potentially released by the interaction of Cd with pulmonary cells (alveolar macrophages; epithelial cells), have been shown to induce MT (22-24). In addition, growth factors released from activated alveolar macrophages could lead to the observed epithelial cell proliferative response (25). Although the increase in cell proliferation could potentially make those cells more vulnerable to a genotoxic Cd effect, the protection given by the increased cellular MT content through binding of Cd may prevent genotoxic interaction of Cd with DNA. This hypothetical scenario could explain both the species differences in inflammation, including cell proliferation, and pulmonary carcinogenesis.

The different inducibility of MT in pulmonary tissues and cells in rats vs. mice is possibly also responsible for differences...
in pulmonary Cd retention. In mice, only 2% of the total pulmonary Cd was retained in the lavaged cells in contrast to 12% in rats, which is in line with the observed greater MT inducibility in rat alveolar macrophages available for Cd binding. In fact, the amount of MT (−10 µg) found in the lavaged free lung cells (1.46 × 10^3) from CdCl₂-exposed rats in our study would suffice to bind the 0.9 µg Cd found in these lavaged cells. Also of interest that the pulmonary retention halftime of cadmium is about 2-fold longer in mice than in rats, i.e., a retention halftime of about 70 days in rats versus ~140 days in mice (data not reported). Data from Heinrich et al. (3) also point to a longer pulmonary retention of Cd in mice than in rats, which might be due partially to the greater MT content in Cd-exposed mouse lungs and binding of Cd. An important conclusion is that a longer retention of Cd in the lung does not necessarily translate into a greater risk for inducing lung tumors; it appears that levels and inducibility of pulmonary MT are crucial factors. Nonhuman primates retain pulmonary Cd about 10-fold longer than rats (26), but this should not be viewed as being at a greater risk for lung tumors than rats since nothing is known about MT levels in primate lungs after Cd exposure.

With respect to pulmonary Cd retention and MT levels, the measured MT content in rat and mouse lungs in our study would not be sufficient to bind all of the retained cadmium. For example, at the end of the 4-week exposure, 22.2 and 7.2 µg MT (Table 4) were found in the rat and mouse lungs, respectively. Even completely saturated with Cd, this would only account for the binding of about 2.2 and 0.72 µg Cd, i.e., for about 30% of the actually retained Cd of 7.4 and 2.2 µg in the lung (Table 1). This implies that in both rats and mice almost the same fraction of the retained pulmonary Cd is not bound to MT but probably is bound to other proteins. However, the fact that pulmonary tissue MT, in particular epithelial MT (Figure 5), is markedly increased in the mice apparently provides sufficient protection of these target cells against a potential carcinogenic Cd effect. As discussed in the preceding section, MT in the lavaged cells of rats is sufficient to bind all Cd present in this fraction. This is also true for MT and Cd in lavaged mouse cells.

Although our studies indicate that the rat did not respond with a significant up-regulation of MT expression in lung tissue after Cd exposure, the result should be seen in relation to the Cd dose. On a per gram lung basis, mice retained about twice as much Cd as rats (Table 1), which can be explained by a greater pulmonary deposition of the inhaled Cd due to the greater ventilation rate in mice per g body mass. Moreover, results of Hart et al. (18) clearly show that increasing doses of pulmonary Cd in rats will also lead to an increased induction of MT in their lungs. We should, therefore, expect to find significant increases in lung MT also in rats when higher Cd doses in the lung are retained. Our future studies will be directed toward establishing dose-response relationships with respect to pulmonary MT induction, inflammation, and cell proliferation in rats and mice to further investigate species differences. However, our present results show that Cd aerosols administered at the same concentration to these two species clearly result in different lung MT inducibility and inflammatory processes. Thus, the important question about the most appropriate animal model for extrapolation to humans needs further attention and could be addressed in studies involving in vitro Cd exposures of human and rodent lung cells and lung tissues through evaluating and comparing MT expression, proliferative effects, and other specific endpoints.

In conclusion, our studies showed that the same inhaled concentration of CdCl₂ resulted in a greater pulmonary inflammatory response, greater cell proliferative response, and greater MT expression in bronchial and alveolar epithelial cells in mice than in rats. Pulmonary retained Cd per gram lung was about 2-fold greater in mice than in rats. Despite the greater inflammatory and cell proliferative response a lower pulmonary carcinogenic effect of inhaled Cd had been observed in mice in previous studies. We suggest that this apparent resistance of mice to pulmonary carcinogenic effects of Cd is due to an increased protection against a genotoxic effect of cadmium by MT induced in respective target cells.

NOTE ADDED IN PROOF:
At the 1993 spring meeting, IARC determined that Cd is carcinogenic to humans.

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