Cadmium-induced inhibition of the growth and metastasis of human lung carcinoma xenografts: role of apoptosis

Michael P. Waalkes2 and Bhalchandra A. Diwan1

Inorganic Carcinogenesis Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute at the National Institute of Environmental Health Sciences, 111 Alexander Drive, PO Box 12233, MD F0-09, Research Triangle Park, NC 27709 and 2Intramural Research Support Program, SAIC Frederick, Frederick, MD 21702, USA

Our previous studies indicate that cadmium in mice can inhibit the formation of chemically induced and spontaneously occurring tumors in the liver and lung. Cadmium is an effective anti-tumor agent when given at non-toxic doses and even when given well after tumor formation, implying a unique sensitivity in certain tumor cells. The present studies tested the ability of cadmium to inhibit growth and progression of transplanted human pulmonary tumor xenografts. Male athymic nude mice were inoculated with either H460 cells, originally derived from a non-small cell pulmonary carcinoma, or DMS 114 cells, originally derived from a small cell lung carcinoma, under the left renal capsule. Starting 1 week later mice received 0, 125 or 250 p.p.m. cadmium in the drinking water, levels without effect on host animal growth or survival, and were observed over the next 4 weeks (H460 cells) or 100 days (DMS 114 cells). An additional experiment gave cadmium as an i.v. loading dose (20 µmol/kg) 4 days after renal inoculation with H460 cells and 200 p.p.m. cadmium in the drinking water from 7 days onward, with an observation period of 28 days. Cadmium caused dose-related reductions in the growth of tumors resulting from the inoculation of either H460 or DMS 114 cells of up to 83%. Additionally, cadmium reduced the rate of tumor metastasis to the lung by up to 58%. Cadmium treatment had no effects on either Bcl-2 or Bax protein expression in tumor xenografts, indicating that apoptotic pathways probably do not contribute to this anti-neoplastic effect. These studies show cadmium can effectively reduce growth and progression of human lung carcinoma xenografts in a fashion that is probably independent of apoptosis.

Introduction

Previously, we found that cadmium treatment could either abolish or substantially reduce N-nitrosodiethylamine (NDEA)-induced and spontaneous tumor formation in the mouse liver or lung (1–3). Cadmium-induced perturbation of cancer development occurred if the metal was either given chronically in the drinking water or given as a single, non-toxic i.v. treatment (1–3). The ability of cadmium to depress pulmonary and hepatic tumor burden in mice was largely independent of the interval between the nitrosamine exposure and the cadmium treatment (2,3). In fact, cadmium treatment could be delayed as long as 32–40 weeks after the nitrosamine, at a time point after tumors were well formed, and still be remarkably effective (2,3). For example, a single, non-toxic i.v. treatment with cadmium given 40 weeks after NDEA exposure reduced hepatic tumor incidence in treated mice by nearly 50%, while the tumors remaining after cadmium exposure were greatly diminished in size (3). The ability to reduce the incidence of both nitrosamine-induced and spontaneous tumor formation indicates that this effect of cadmium is based on a characteristic of the tumor itself rather than as a function of inducing agent. In this regard, when tumor-bearing livers were microscopically analyzed shortly after cadmium treatment (i.e. <1 week) it was apparent that the metal had caused extensive cellular necrosis specifically within the hepatic tumors, while preserving the surrounding normal tissue (3). Thus, cadmium can be effective as an anti-tumor agent in mice even when given well after tumors were formed through what appears to be a tumor-specific effect (3). Cadmium-induced tumor suppression could be accomplished with doses that were not overtly toxic (3), which would be a positive attribute for any cancer chemotherapeutic. Further study has shown that metallothionein, a protein associated with cadmium tolerance, is poorly expressed in liver and lung tumors from mice (3), an observation that may account for the tumor cell selectivity of cadmium in mice. Human hepatocellular carcinomas (3) and pulmonary small cell carcinomas (4) also typically show a poor expression of metallothionein, possibly predicting a common sensitivity to cadmium. The chemotherapeutic potential of cadmium deserves further study.

Recent evidence indicates that cadmium may induce cell death, at least in part, through apoptosis (5–9). For example, cadmium treatment effectively induces apoptosis in cell systems in vitro (5–7) as well as in several target tissues in vivo, such as the liver and the urinogenital organs (8,9). In this regard, the Bcl-2 family of proteins is thought to be important in apoptosis (10–13). The Bcl-2 family consists of both pro-apoptotic members, such as Bax, and anti-apoptotic members, such as Bcl-2 (10–13). The ratio of anti-apoptotic to apoptotic forms appears to dictate the response to a death stimulus (10–13), although both Bcl-2 and Bax can regulate apoptosis independently of the other (14). Bcl-2 and Bax expression have been reported in a variety of tumors (15–21), including non-small cell and small cell pulmonary carcinomas. Although there is some association between cadmium treatment and apoptosis (5–9), the role of apoptosis in cadmium-induced tumor suppression is unknown.

The present study is a continuation of our earlier studies (1–3) that uses human tumor cell xenografts in athymic nude mice as a model and investigates the effects of cadmium on the growth and metastasis of human tumors. Such xenograft systems have been widely used to study the growth and progression of a variety of tumors and to investigate potential chemotherapeutics (22–29). Since cadmium has proven effective against pulmonary tumor formation in mice, the

Abbreviation: NDEA, N-nitrosodiethylamine.

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Materials and methods

Chemicals
Cadmium chloride (CdCl₂) was purchased from Sigma (St Louis, MO). Polyclonal rabbit anti-Bcl-2 and anti-Bax antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Tumor cells
H460 cells were originally derived from a human non-small cell pulmonary carcinoma. Both cell types were obtained from the National Cancer Institute Repository (Frederick, MD) and cultured under standard conditions in RPMI 1640 medium to the required number for inoculation. Cadmium, as CdCl₂, was dissolved in saline for i.v. injection or in acidified water at 0, 125 or 250 p.p.m. for inoculation (10 ⁶ cells in a total volume of 25 l) under the left renal capsule on experimental day 0 and 4. Animals then received cadmium per os at a constant rate of 1×10⁶ cells in a total volume of 25 µl under the left renal capsule. The right kidney was untreated and served as the control for measuring tumor growth. After 7 days the mice received CdCl₂ per os at either 0, 125 or 250 p.p.m. cadmium. These levels of oral cadmium were without effect on growth or survival (Table I). After tumor inoculation mice were observed over the next 28 (H460 cells) or 100 (DMS 114 cells) days. In a separate experiment, H460 cells were inoculated (1×10⁶ cells) into nude mice with and without cadmium treatment.

Immunohistochemistry
For determination of the expression of Bcl-2 and Bax proteins, selected sections (seven each) of tumors resulting from the inoculation of either H460 or DMS 114 cells were evaluated. The sections were microwaved after deparaffinization for 10 min in citrate buffer. Immunohistochemical detection was performed with polyclonal rabbit anti-Bcl-2 and anti-Bax antiserum (1:200). Reactions were visualized with avidin–biotin–peroxide kit (Vector-Stain Elite Kit; Vector Laboratories, Burlingame, CA) with diaminobenzidine as the chromogen.

Results

Initial studies investigated the toxic effects of oral cadmium to define the non-toxic range for use in xenograft studies (Table I). Cadmium in the drinking water over a 28 day period did not induce overt toxicity until the 400 p.p.m. level was reached. At levels of 400 p.p.m., the metal had no effect on lethality or on final body weights. Thus, doses of 250 p.p.m. or less were selected for all further study.

The effects of oral cadmium (125 and 250 p.p.m.) on the growth and metastases of H460 xenografts injected under the renal capsule were then studied. Mice were inoculated with H460 cells on day 0 and received cadmium per os starting 7 days later. The experiment ended 28 days after inoculation. Cadmium treatment did not alter the incidence of tumors arising at the site of inoculation (Table II). Oral cadmium did, however, cause a marked, dose-related reduction in tumor mass. In fact, at the highest cadmium dose tumor weight was reduced by 45% (P 0.05). The metastatic rate of tumors appeared to be reduced in a cadmium dose-related fashion, although this was not statistically significant (P 0.22).

The effects of oral cadmium (125 and 250 p.p.m.) on the growth and metastases of DMS 114 xenografts are shown in Table II. Mice were inoculated with DMS 114 cells under the renal capsule on day 0 and received cadmium per os starting 7 days later. At 100 days after inoculation, cadmium treatment did not alter the incidence of tumors arising at the site of DMS 114 cell inoculation (Table III). However, a marked, dose-related reduction in tumor mass occurred with cadmium exposure. At the highest level of cadmium tumor weight was reduced by 80% (P 0.05). Additionally, the metastatic rate of the tumors resulting from DMS 114 inoculation showed a significant (P 0.05) trend in reduction based on cadmium dosage to levels 50% of the control rate.

In further experiments the effects of an i.v. loading dose of cadmium prior to oral cadmium exposure on H460 xenografts

Table I. Preliminary toxicity testing of oral cadmium in NCr-nu mice

<table>
<thead>
<tr>
<th>Dose (p.p.m.)</th>
<th>No. of survivors/ no. treated</th>
<th>Final body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10/10</td>
<td>31.1 ± 0.8</td>
</tr>
<tr>
<td>50</td>
<td>10/10</td>
<td>30.5 ± 1.1</td>
</tr>
<tr>
<td>100</td>
<td>10/10</td>
<td>29.9 ± 0.8</td>
</tr>
<tr>
<td>200</td>
<td>10/10</td>
<td>28.8 ± 0.7</td>
</tr>
<tr>
<td>400</td>
<td>8/10</td>
<td>27.1 ± 1.2</td>
</tr>
<tr>
<td>600</td>
<td>7/10</td>
<td>25.9 ± 0.9</td>
</tr>
<tr>
<td>800</td>
<td>7/10</td>
<td>24.0 ± 0.8</td>
</tr>
<tr>
<td>1000</td>
<td>6/10</td>
<td>24.9 ± 1.0</td>
</tr>
</tbody>
</table>

*a* Cadmium, as CdCl₂ was added to the drinking water at the levels designated starting at day 0 for 28 days.

*b* Body weight at the conclusion of the experiment in surviving animals.

Given as mean ± SEM.

Significantly (P < 0.05) different from control.

Table II. Effect of oral cadmium on the growth and metastasis of H460 human tumor xenografts in NCr-nu mice

<table>
<thead>
<tr>
<th>Dose (p.p.m.)</th>
<th>No. of tumors/ no. treated</th>
<th>Mean tumor weight (g)</th>
<th>Metastatic rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9/9</td>
<td>2.54 ± 0.43</td>
<td>5/9</td>
</tr>
<tr>
<td>125</td>
<td>9/9</td>
<td>1.59 ± 0.33</td>
<td>3/9</td>
</tr>
<tr>
<td>250</td>
<td>10/10</td>
<td>1.36 ± 0.19</td>
<td>2/9</td>
</tr>
</tbody>
</table>

*a* Tumor cells were inoculated on day 0 under the left renal capsule.

Cadmium was added to the drinking water at the levels designated starting at day 7. All animals were killed by 28 days.

*b* Data expressed as means ± SEM (n = 9).

*c* Exclusively pulmonary metastases. A test for trend indicated a trend based on cadmium dose that approached significance (P = 0.22).

*d* Significant (P < 0.05) differences between treated and control.

For determination of the expression of Bcl-2 and Bax proteins, selected sections (seven each) of tumors resulting from the inoculation of either H460 or DMS 114 cells were evaluated. The sections were microwaved after deparaffinization for 10 min in citrate buffer. Immunohistochemical detection was performed with polyclonal rabbit anti-Bcl-2 and anti-Bax antiserum (1:200). Reactions were visualized with avidin–biotin–peroxide kit (Vector-Stain Elite Kit; Vector Laboratories, Burlingame, CA) with diaminobenzidine as the chromogen.

Data analysis
A level of P < 0.05 in two-sided testing was considered significant in all cases. With all incidence data a Fisher’s exact test was used. Student’s t-test or Durnell’s t-test after analysis of variance were used for tumor size or volume comparisons, as appropriate. The χ² test for trend was used to define trends associated with cadmium dose for incidence data.
received a single i.v. dose of cadmium (20 µmol/kg) at 4 days and cadmium per os (200 p.p.m.) from 7 to 28 days. The experiment ended 28 days after inoculation. Tumor weight (left) is given in mg and tumor volume (right) is given in mm³. Values represent means ± SEM (n = 7).

Table III. Effect of oral cadmium on the growth and metastasis of DMS 114 human tumor xenografts in NCr-nu mice

<table>
<thead>
<tr>
<th>Dose (p.p.m.)</th>
<th>No. of tumors/no. treated</th>
<th>Mean tumor weight (mg)</th>
<th>Metastatic rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7/10</td>
<td>884 ± 252</td>
<td>7/7</td>
</tr>
<tr>
<td>125</td>
<td>7/10</td>
<td>295 ± 88</td>
<td>3/7</td>
</tr>
<tr>
<td>250</td>
<td>7/10</td>
<td>147 ± 26</td>
<td>3/7</td>
</tr>
</tbody>
</table>

*Tumors were inoculated on day 0 under the left renal capsule. Cadmium was added to the drinking water at the levels designated starting at day 7. All animals were killed by 100 days.

were assessed. Mice were inoculated with H460 cells under the renal capsule on day 0 and received i.v. cadmium at concentrations of 1.0 µM prior to s.c. inoculation in nude mice, there was a marked suppression of tumor growth and progression in vivo after inoculation (32).

Furthermore, when cells derived from viral-induced MSB sarcomas were inoculated into mice, subsequent oral exposure to cadmium very effectively blocked tumor growth and markedly enhanced the rate of tumor regression (33). Cadmium treatment can also enhance the survival of mice inoculated with plasmacytoma cells (34). Cadmium, when given by i.p. injection 5 weeks after the s.c. inoculation of Dunn osteosarcoma cells, reduces growth and causes necrosis of the resulting sarcomas (35). With liver tumors elicited by nitrosamine exposure in mice cadmium treatment also induced tumor-specific necrosis while preserving the normal surrounding tissue (3). These findings together support a mechanism involving a direct effect of cadmium on tumor cells as a means of reduced tumor growth and progression.

There are several possible processes by which cadmium could perturb tumor development. Cadmium has been shown to induce apoptosis in some cells and tissues (5–9) and this

Discussion

The implantation of human tumor fragments or cells under the renal capsule of rodents is widely used as a model to test the effectiveness of chemotherapeutic agents for a variety of tumor types (22–27) and to study metastatic potential (28,29). The effectiveness of agents in such renal inoculation systems has been shown on many occasions to be prognostic of actual clinical efficacy. In the present study, both a regimen of oral cadmium alone or a combination of i.v. and per os cadmium proved very effective in blocking the growth and reducing the metastatic potential of tumors resulting from human pulmonary carcinoma cell lines which had been inoculated under the renal capsule of immunodeficient mice. Cadmium markedly reduced the growth of tumors resulting from either the inoculation with H460 or DMS 114 cancer cells. The most dramatic reductions occurred with tumors derived from what was originally a small cell pulmonary carcinoma (DMS 114 cells) and these reductions exceeded 80% of the tumor mass in some instances. Cadmium treatment also suppressed metastatic rate of the resulting tumors in several cases. Although in vivo cadmium can be very toxic, causing a variety of acute and chronic lesions (30,31), the effectiveness of cadmium in suppression of tumor growth in this study was not associated with any overt systemic toxic effects of the metal.

The dramatic reduction in the growth of human lung carcinoma xenografts in the present study is consistent with the previously reported efficacy of cadmium against spontaneously occurring or nitrosamine-induced pulmonary and hepatic tumors in mice (1–3). Other studies have indicated that when the normally tumorigenic rat L6 myoblast cells are exposed to cadmium in vitro at concentrations of 1.0 µM prior to s.c. inoculation in nude mice, there was a marked suppression of tumor growth and progression in vivo after inoculation (32).
could be a factor in reducing tumor burden. However, the findings of cadmium-induced apoptosis are not consistent and cadmium can in fact block apoptosis in some instances (36). The lack of effect of cadmium on either Bcl-2 or Bax expression in the present work strongly suggests that the apoptotic pathway of cell death does not significantly contribute to the anti-neoplastic effects of cadmium in tumors resulting from inoculation of either H460 or DMS 114 cells. The observation that liver tumors in mice undergo necrosis after cadmium treatment (3) would also argue against the stimulation of programmed cell death as a primary mechanism. Although cadmium did not induce overt necrosis in the xenograft tumors in this study this does not eliminate the possibility that cytotoxic cell death plays a major role in the tumor suppressive effects. In some cases cadmium may suppress the expression of genes associated with cellular proliferation (37) and can be associated with a cell cycle arrest (38). There is also clear evidence that cadmium can be very cytotoxic in vitro (2,30,39,40) and direct cytotoxicity would be consistent with the finding of necrosis in liver tumors (3) or sarcomas (35). However, the capacity of in vitro cadmium exposure prior to inoculation to perturb the growth and progression of tumors arising after inoculation of L6 myoblasts (32) could not be mediated through direct cytotoxicity resulting in tumor cell necrosis. Therefore, it is possible that multiple mechanisms, including direct cytotoxicity, could play roles in cadmium-induced tumor suppression.

It appears that some tumors may have a heightened sensitivity to cadmium. The cytotoxic impact of cadmium is often a function of the level of cellular metallothionein, a cadmium-inducible metal-binding protein encoded by the MT gene which sequesters cadmium and thereby mitigates its toxicity (41,42). As a general rule the less metallothionein present the less a cell or tissue is resistant to cadmium-induced cytotoxicity (41,42). Our studies also show that metallothionein is poorly expressed in mouse liver and lung tumors (2,3), as well as in human hepatocellular carcinomas (3). Other work indicates metallothionein is poorly expressed in human non-small pulmonary carcinomas under normal circumstances (4). In a study of the comparative cadmium cytotoxicity in non-transformed and transformed (tumorous) liver cells (43), HepG2 cells (derived from a human hepatocellular carcinoma) were hypersensitive to cadmium in comparison with non-transformed liver cells (TRL 1215). This cellular hypersensitivity was associated with much lower basal levels of metallothionein and a minimal induction of metallothionein after cadmium exposure in the HepG2 cells (43). When the TRL 1215 cells undergo spontaneous transformation to a recognizable malignant phenotype, there is a marked suppression of the basal expression of the MT gene (44). The transformation of TRL 1215 cells is also accompanied by an enhanced sensitivity to cadmium-induced cytotoxicity, very likely due to diminished MT expression (3,43). Generally speaking, pulmonary tissue has a relatively low basal metallothionein level which is not highly induced by cadmium exposure (45,46). Therefore, the hypersensitivity of liver tumors, and possibly of lung tumors, may be due to poor expression of metallothionein in the tumor cells.

Cadmium is classified as a known human carcinogen (31) and is highly toxic (30,39,40), with a very long biological half-life (31), all of which would be clear drawbacks to any pharmaceutical application. However, as a class of agents anti-neoplastic drugs generally have a very narrow therapeutic index with a greater potential for harmful side-effects than most other categories of pharmaceuticals (47). In fact, many cancer chemotherapeutics are also potential human carcino-
Cadmium-induced inhibition of carcinoma xenografts
gens, including cisplatin, nitrogen mustards, procarbazine and
the combination chemotherapeutic regimen MOPP (48). The
list of chemotherapeutic carcinogens includes several widely
used and effective drugs. A certain level of risk is accepted
with the use of any drug and the level of acceptable risk is
linked to the gravity of the untreated disease.

In summary, cadmium can dramatically reduce human
pulmonary carcinoma growth and progression in xenograft
model systems. These tumor-suppressive effects of cadmium
occur with non-toxic doses, indicating a heightened sensitivity
of tumor cells to the metal. Lack of effect of cadmium on
some key proteins in the apoptotic pathway suggests that
apoptotic cell death is not responsible for the anti-neoplastic
effects of cadmium in these tumors. It will be of interest to
test cadmium against hepatocellular carcinoma xenografts and
possibly in combination with other cancer chemotherapeutics.

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References


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