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Effect of 3-aminotriazole on anchorage independence and mutagenicity in cadmium- and lead-treated diploid human fibroblasts

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Cadmium and lead have been shown to induce cellular transformations and gene mutations in cultured rodent cells, as well as tumours in live animals. However, the mechanisms by which these metals cause cellular transformations and mutations in human cells have not been explored. In this study, we investigated the abilities of cadmium and lead to induce anchorage-independent transformations and *hprt* gene mutations in diploid human fibroblasts. Human fibroblasts were exposed to either cadmium acetate (0–60 μM) or lead acetate (0–2 mM) for 24 h. After removal of the metals, the cells were kept in exponential growth for 7 and 9 days before mutation and anchorage-independence assays were taken, respectively. Both cadmium and lead significantly induced anchorage-independent colonies in dose-dependent manners; the frequencies of anchorage-independent colonies induced by these metals were similar to those induced by *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine at approximately equal cytotoxic dose ranges (30–10% survival). 3-Aminotriazole at non-cytotoxic dosages decreased catalase activity by >80%, and markedly enhanced cadmium-induced cytotoxicity and anchorage-independent colonies. Cadmium uptake by human fibroblasts was not affected by 3-aminotriazole co-administered with 10 μM of cadmium; whereas cadmium uptake and accumulation were enhanced 1.5-fold by 3-aminotriazole co-administered with 1–2.5 μM of cadmium. Lead-induced anchorage-independence or cytotoxicity was not affected by 3-aminotriazole co-treatment; however, 3-aminotriazole did significantly enhance lead uptake and accumulation in human fibroblasts. Neither cadmium- nor lead-induced 6-thioguanine-resistant mutation frequency in human fibroblasts. Co-administering these metals with 3-aminotriazole did not enhance mutations in human fibroblasts. These results suggest that cadmium and lead may both act as tumour promoters in diploid human fibroblasts, and that reactive oxygen species is more important in cadmium- than lead-induced cytotoxicity and anchorage-independence.

Introduction

Cadmium and lead compounds are ubiquitous environmental contaminants that have been evaluated as potential human carcinogens (1–4). Cd induces morphological transformations, chromosomal aberrations and mutagenicity in rodent cells (5–8). A significant body of evidence confirms cadmium carcinogenicity in animal models; e.g. cadmium has been shown to induce tumours in testes, lungs, prostates, hematopoietic systems, and at injection sites (9,10). Similarly, lead compounds have also been shown to induce renal tumours in rats and mice (11–13), as well as morphological transformations, sister-chromatid exchanges and mutagenicity in cultured rodent cells (14–22).

Metal genotoxicity and carcinogenicity are rather complex because they interact with both DNA and proteins. Cadmium interactions with nucleic acids favor bases over DNA phosphates (23–25); whereas, lead interacts with the phosphate backbone (26). Moreover, it has been reported that these metals can inhibit DNA replication and decrease the fidelity of DNA polymerases (27–29). Both cadmium and lead interact with proteins, particularly at the sulph-hydryl group (12,30). They have been reported to disturb calcium metabolism and may act as antagonists to zinc and iron (12,13,31). Cadmium and lead induce the expression of several genes, such as those that encode metallothioneins, heme oxygenases, glutathione transferases, heat-shock proteins, acute-phase reactants and DNA polymerase β (32–36). Moreover, cadmium and lead stimulate cellular signal transduction pathways, and thus may act as tumour promoters (37–40).

Intracellular reactive oxygen species (ROS*) have been implicated as important mediators of chemical genotoxicities and carcinogenicities (41,42). DNA strand-breakage, chromosomal aberrations and mutations induced by cadmium can be suppressed by ROS scavengers, implying that ROS mediate cadmium genotoxicity (8,43,44). Cadmium also alters ROS amounts and antioxidant enzyme activities in rat testicular Leydig cells (45). Lead-induced sister-chromatid exchanges are inhibited by ascorbic acid suggesting that free-radical defence pathways may influence lead genotoxicity (46). These metals thus play direct or indirect roles in genotoxicity and carcinogenicity.

The epidemiological evidence for lead carcinogenicity in humans is still inadequate, and that for cadmium carcinogenicity is complicated by confounders (1–4). Based primarily on rodent data, cadmium and lead are rated as group-1 and group-2B carcinogens, respectively (2,4). However, the tendencies of these metals to induce human cell-transformations and mutations remain unexplored. Transformation and mutagenesis mechanisms may differ in human and rodent cells, e.g. human fibroblasts are more resistant than rodent fibroblasts to chemical transformation and may independently express different transformation phenotypes in different sequential orders than do rodent fibroblasts (47,48). It is therefore important to study transformation and mutagenicity of human cells induced by chemicals implicated as human carcinogens. Anchorage independence, i.e. cells' abilities to grow in semi-solid media, has been considered a genetic marker because the anchorage-independent phenotype was stable, and cells exposed to carcinogens exhibit dose-dependent

***Abbreviations:** ROS, reactive oxygen species; *hprt*, hypoxanthine (guanine) phosphoribosyltransferase; 3-AT, 3-aminotriazole; PBS, phosphate-buffered saline; 6-TG, 6-thioguanine.

increases in the frequency of anchorage-independent colonies (47–49). In this study, we explored whether cadmium or lead could transform diploid human fibroblasts into anchorage-independent colonies, as well as induce mutations at the hypoxanthine (guanine) phosphoribosyltransferase (*hprt*) gene in these cells. Additionally, the role of ROS in the cytotoxicity, anchorage-independent growth and mutagenic activities of these metals was investigated by co-administering metals with 3-aminotriazole (3-AT), an inhibitor of catalase (8), peroxidase (50), cytochrome p450IIE1 (51) and heme biosynthesis (52) to provide more thorough information about metal genotoxicity and transformation mechanisms.

Materials and methods

Cell culture

Diploid human fibroblasts HFW were established from the foreskin of a Chinese infant. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies Co., Grand Island, NY) supplemented with sodium bicarbonate (0.37%, w/v), L-glutamine (0.03%, w/v), penicillin (100 units/ml), streptomycin (100 μg/ml) and foetal calf serum (10%, w/v; Biological Industries, Kibbutz Beth Haemek, Israel). Cell cultures were maintained at 37°C in a humidified incubator containing 10% CO_2 in air. HFW cells used in this study have been checked to be mycoplasma-free using the Mycoplasma PCR$^+$ Primer Set (Stratagene, La Jolla, CA).

Cytotoxicity assay

Cells in exponential growth were trypsinized and 1×10^6 cells were plated onto a 100-mm Petri dish 16–18 h before metal treatment. Cadmium acetate (⩾99% purity, Cat. No. 2003, Merck, Darmstadt, Germany) and lead acetate (⩾99.5% purity, Cat. No. 7375, Merck) were dissolved in MilliQ-purified water (Millipore, Bedford, MA). Cells were treated with either cadmium or lead for 24 h in a complete medium. In experiments to determine the effect of ROS on metal-induced cytotoxicity, transformation and mutagenicity, cells were incubated with 80 mM of 3-AT (Sigma, St Louis, MO) for 1 h before co-exposure to either cadmium or lead for 24 h. *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG; Sigma) was used as a positive control; cells were exposed to 10 μM of MNNG for 1 h in Hepes-buffered (15 mM) serum-free DMEM. At the end of treatment, the drug-containing medium was removed, and cells were washed twice with phosphate-buffered saline (PBS) before trypsinization. Cells were diluted and triply plated at densities of 100–1000 cells/100-mm Petri dishes. The cells were cultured for 14 days with one fresh media replacement and stained with 1% crystal violet solution. Colony numbers were counted for cytotoxicity determination which was calculated to be the number of colonies in the treated cells divided by the number of colonies obtained from untreated cells (8,21).

Anchorage-independence assay

The cadmium-, lead- and MNNG-treated and untreated cells described in the section on cytotoxicity assay were maintained in exponential growth for 9 days before anchorage-independence assay by a modification of previous methods (49,53). A total of 10^6 cells per individual population were assayed at 10^5 cells/60-mm dish. The growth medium for anchorage-independence assay consisted of DMEM containing Bacto-agar (Difco, Detroit, MI) (top-agar and bottom-agar were 0.33 and 0.5%, respectively), sodium bicarbonate (0.37%, w/v), L-glutamine (0.03%, w/v), penicillin (100 units/ml), streptomycin (100 μg/ml), non-essential amino acid (0.1 mM), b-fibroblast growth factor (5 ng/ml; Sigma) and tryptose phosphate broth (0.22%, w/v). The cells (10^5), suspended in 3 ml of top-agar medium, were allowed to solidify on top of a bottom-agar layer (5 ml) and were cultured for 4 weeks at 37°C in a humidified incubator containing 5% CO_2 in air; 0.5 ml of agar-free growth medium was added to the top-agar layer every week to compensate for evaporation. After 4 weeks, the cells were stained with iodonitrotetrazolium violet (1 mg/ml in MilliQ water; Sigma) and colonies >60 μm in diameter were scored using a dissecting microscope equipped with a transmitted light source (model SMZ-U, Nikon, Tokyo). An aliquot of the cell suspension used in the top-agar for anchorage-independence assay was diluted with agar-free culture medium, plated onto 100-mm Petri dishes (100 cells/dish), incubated for 2 weeks and stained with 1% crystal violet to determine the replating efficiency of the cells. This value was used to determine cell viability in the anchorage-independence assay.

Mutagenicity assay

The cadmium-, lead- and MNNG-treated and untreated cells were maintained in exponential growth for 7 days to allow for expression of resistance to 6-thioguanine (6-TG). One million cells from each individual treatment were plated onto thirty 100-mm Petri dishes in a selective medium containing 40 μM of 6-TG. Macroscopic 6-TG resistant clones were developed 2 weeks after culturing at 37°C in a 10% CO_2 incubator. Plating efficiency of cells at the time of selection was also assayed in a non-selective medium to correct for the observed mutation frequency. The mutation frequency was calculated to be $-\ln(P_0)$ divided by the average number of viable cells per culture, where P_0 is the proportion of dishes with no mutants (21).

Determination of cellular cadmium and lead levels

Cells (1×10^6) were treated or not treated with 80 mM of 3-AT for 1 h, and subsequently exposed to either cadmium or lead at various concentrations in media containing serum for 24 h. In the metal-accumulation experiments, cadmium- or lead-treated cells were allowed to incubate for another 8 h after removal of metals from the media. At the time of harvesting, cells were washed 4–6 times with PBS, trypsinized and the numbers of cells were determined. Next, cells were centrifuged and the cell pellet was sonicated in MilliQ-purified water. Total metal concentration was analysed using a polarized Zeeman atomic absorption spectrophotometer (Spectr AA-30, Varian) equipped with an autosampler and a graphite furnace. The analytical conditions for cadmium concentrations were set as follows: absorption wavelength 228.8 nm, lamp current 4 mA, and atomizing temperature of 1800°C; for lead concentrations: absorption wavelength 283.3 nm, lamp current 5 mA and atomizing temperature of 2100°C.

Determination of catalase activity

Cells were washed four times with PBS after metal treatments and harvested using a rubber policeman. The cell suspension was centrifuged at 1000 rpm for 5 min at 4°C. The cell pellets were sonicated in 0.5 ml of 100 mM phosphate buffer (pH 7.0) and the debris was removed by centrifuging at 10 000 rpm for 30 min at 4°C. The supernatant was then used for determination of catalase activity and protein content. Catalase activity was measured as described by Aebi (54). Assay was performed at room temperature in a 1-ml mixture containing clear cell lysate, 100 mM of phosphate buffer (pH 7.0) and 10 mM of H_2O_2. The decomposition of H_2O_2 was followed directly by a decrease in absorbance at 240 nm. Protein concentrations were determined using bovine serum albumin as a standard. Enzyme activity was expressed in μmol of H_2O_2 decrease/min/mg protein.

Statistical analyses

Data obtained from cytotoxicity, anchorage-independence, catalase activity and cellular metal level assays were analysed using one-way analysis of variance followed by Duncan's multiple range test or two-way analysis of variance with a statistical package for social science software. Mutation frequency was assessed using the Poisson distribution test. The level of significance was set at $P < 0.05$.

Results

3-AT did enhance cadmium-induced cytotoxicity and anchorage independence

Diploid human fibroblasts HFW were exposed to 0–60 μM of cadmium acetate for 24 h and cytotoxicity was determined by colony-forming ability assay immediately after treatment. Figure 1a shows that 30 μM of cadmium acetate was required to reduce the survival of treated cells to 37% (D_0). Cells treated and not treated with cadmium were maintained in exponential growth for 9 days, i.e. they were subcultured two or three times, before determination of their transformation to anchorage independence. The frequency of anchorage-independent colonies per 10^6 viable cells obtained from untreated HFW fibroblasts was 101.3 ± 9.9 (18 individual experiments). One-way analysis of variance followed by Duncan's multiple range test showed that cadmium acetate significantly induced anchorage-independent colonies in a dose-dependent manner. The average Petri-dish replating efficiencies of cells subjected to anchorage-independence assay in untreated and cadmium-treated populations were 62.2 ± 4.4 and 45.6 ± 3.5%, respectively. This indicates that the cell viability of treated cells at the selection time was similar to that of untreated cells.

To explore the role of ROS in cadmium cytotoxicity and

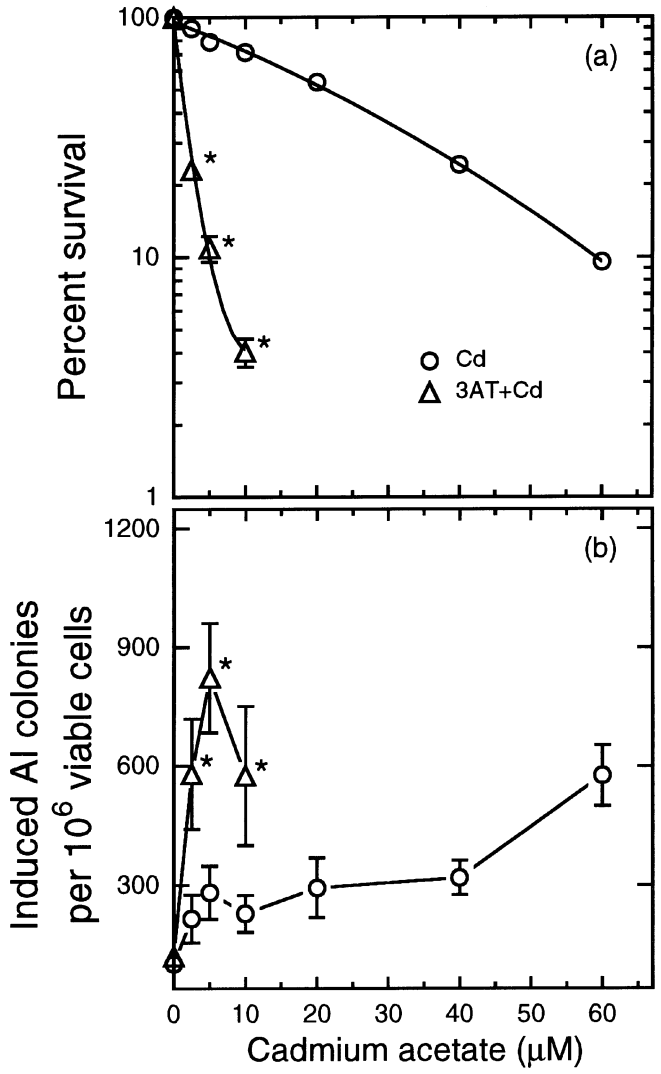

Fig. 1. Effects of 3-AT on Cd-induced cytotoxicity (**a**) and anchorage-independent colonies (**b**) in human diploid fibroblasts. Cells were treated (open triangle) or not treated (open circle) with 80 mM of 3-AT for 1 h and then treated with cadmium acetate for 24 h. The cells were subjected to cytotoxicity and anchorage-independence assays as described in 'Materials and methods'. The replating efficiencies of 3-AT-treated and untreated control cells obtained during cytotoxicity assays were 56 ± 3.3% (n = 23) and 60 ± 3.0% (n = 33), respectively. Each anchorage-independence assay was accompanied by a cytotoxicity assay; at least four independent experiments were performed for each dosage level. Bars in the curves denote population SE. Asterisks represent statistically differences between cells treated with cadmium alone and 3-AT plus cadmium.

transformation ability, HFW fibroblasts were exposed to 80 mM of 3-AT for 1 h and then co-treated with cadmium acetate for 24 h. Figure 1 shows that 3-AT markedly enhanced cytotoxicity and numbers of anchorage-independent colonies induced by cadmium; at 5 μM of cadmium, 3-AT reduced the percentage of survival from 79 to 11, and enhanced the number of cadmium-induced anchorage-independent colonies from 281.5 to 822.7 per 10^6 viable cells. 3-AT co-administration also reduced cells' growth rate; however, all the treated cells can be subcultured at least twice before anchorage-independence assay. 3-AT treatment alone only slightly reduced the colony-forming ability (95.5 ± 4.8%; 24 individual experiments); the number of anchorage-independent colonies per 10^6 viable cells obtained from 3-AT-treated cells was 120.6 ± 21.0 (8 individual experiments), similar to that of untreated controls. Two-way analysis of variance showed that co-administering 3-AT significantly enhanced cytotoxicity and the numbers of anchorage-independent colonies induced by 2.5–10 μM of cadmium. MNNG was chosen as a positive control for comparison with the transformation ability of cadmium. Exposing HFW fibroblasts to 10 μM of MNNG for 1 h reduced their colony-forming ability to 25.8 ± 1.3% of that of untreated cells (25 individual experiments); this treatment induced 353.1 ± 65.0 anchorage-independent colonies per 10^6 viable cells (eight individual experiments). The average Petri-dish replating efficiencies of cells subjected to anchorage-independence assay in 3-AT-, 3-AT plus cadmium- and MNNG-treated populations were 48.5 ± 5.7, 33.5 ± 3.8 and 47.3 ± 5.7%, respectively.

3-AT did not alter cytotoxicity and anchorage independence induced by lead acetate

Figure 2 shows the survival and the anchorage independence of HFW fibroblasts exposed to 0–2 mM of lead acetate for 24 h. The D_0 dose of Pb was estimated to be 0.8 mM (Figure 2a). Anchorage-independent colonies increased in linear proportion when lead dosage was increased; at 1.5 and 2 mM of lead, the number of induced anchorage-independent colonies per 10^6 viable cells was 182.6 ± 16.9 and 262.7 ± 37.8, respectively (Figure 2b). One-way analysis of variance followed by Duncan's multiple range test showed that lead acetate significantly induced anchorage-independent colonies in a dose-dependent manner. The average Petri-dish replating efficiencies of cells subjected to anchorage-independence assay in lead- and 3-AT plus lead-treated populations were 67.3 ± 3.5 and 66.9 ± 2.2%, respectively. Two-way analysis of variance showed that 3-AT co-treatment did not cause any significant changes in lead cytotoxicity, nor did it alter the number of lead-induced anchorage-independent colonies. Additionally, the growth rate of cells treated with 0.5–2.0 mM of lead acetate, or co-administered with 3-AT was similar to (0.5–1.5 mM) or slightly slower (2.0 mM) than that of untreated cells.

3-AT inhibited intracellular catalase activity in untreated, cadmium- and lead-treated HFW cells

Intracellular catalase activity was assayed immediately after exposing cells to 3-AT, cadmium, lead, or metal plus 3-AT. The endogenous catalase activity of HFW fibroblasts was 6.42 ± 0.59 μmol H_2O_2/min/mg protein. Exposing HFW fibroblasts to 80 mM of 3-AT for 25 h reduced the intracellular catalase activity to 15.6% of the untreated level (Figure 3). This result confirmed the expectation that 3-AT blocks catalase activity. Intracellular catalase activities in cells treated with 3-AT plus cadmium (40 μM) and 3-AT plus lead (1.5 mM) were 13.7% and 17.3% of the untreated level, respectively (Figure 3). One-way analysis of variance followed by Duncan's multiple range test showed that 3-AT alone and 3-AT plus metals significantly inhibited catalase activity. Furthermore, intracellular catalase activities in HFW fibroblasts treated with either 20–60 μM of cadmium or 0.5–2.0 mM of lead for 24 h were similar to the untreated level (Figure 3).

3-AT did not enhance the weak mutagenicity of cadmium and lead in HFW cells

HFW fibroblasts treated and not treated with cadmium were maintained in exponential growth for 7 days and then assayed for resistance to 6-TG. Mutagenicity experiments were conducted on at least nine individual treatments with a total of

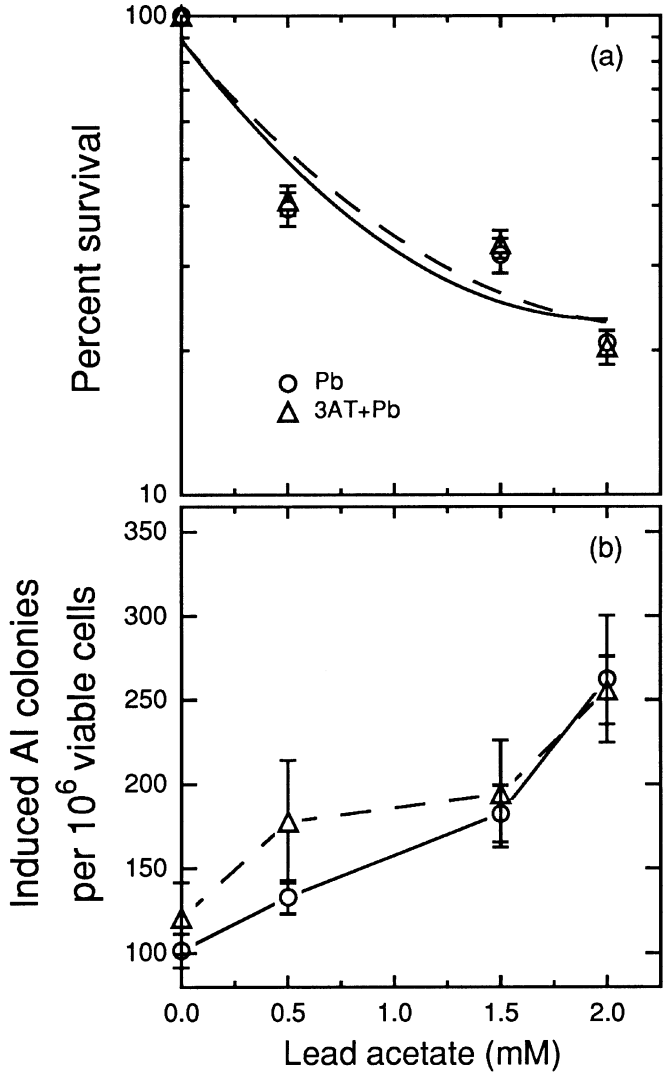

Fig. 2. Effects of 3-AT on Pb-induced cytotoxicity (**a**) and anchorage-independent colonies (**b**) in human diploid fibroblasts. Cells were treated (open triangle) or not treated (open circle) with 80 mM of 3-AT for 1 h and then treated with lead acetate for 24 h. The cells were subjected to cytotoxicity and anchorage-independence assays as described in 'Materials and methods'. Each anchorage-independence assay was accompanied by a cytotoxicity assay; at least four independent experiments were performed for each dosage level. Bars in the curves denote population SE.

5×10^6 clonable cells assayed per cadmium dose. Poisson distribution test indicated that 6-TG-resistant mutation frequencies observed in cadmium-treated cells were not significantly different from those obtained in untreated cells (Table I). In contrast, the 6-TG-resistant mutation frequency of HFW cells was significantly induced by 10–12 μM of MNNG (Table I); the MNNG-induced mutation frequency is consistent with our previous report (55). 3-AT co-treatment, although markedly inducing cadmium cytotoxicity and transformation, did not enhance the weak mutagenicity of cadmium in HFW fibroblasts (Table I).

Table II shows that 0.5–2 mM of lead acetate did not induce mutation frequencies above background levels in HFW fibroblasts; the total clonable cells assayed in each dose was $4–5\times10^6$, obtained from eight or nine individual treatments. The mutation frequencies observed in cells co-administered 3-AT with lead acetate also did not enhance the weak mutagenicity of lead in HFW fibroblasts (Table II).

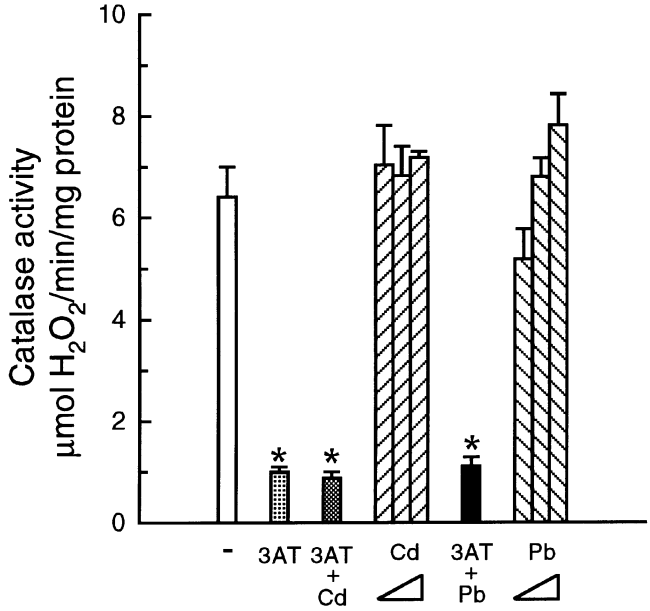

Fig. 3. Intracellular catalase activities in untreated, and Cd, Pb and/or 3-AT-treated human diploid fibroblasts. Cells in exponential growth were treated or not treated with 80 mM of 3-AT for 1 h. Next, cells were treated or not treated with 40 μM of cadmium acetate or 1.5 mM of lead acetate for 24 h. Catalase activities in cells treated with increased doses of Cd (20, 40 and 60 μM) or Pb (0.5, 1.5 and 2 mM) for 24 h were also determined. Catalase activity in cells immediately after treatment was assayed as described in 'Materials and methods'. The results were obtained by averaging four experiments and the bars denote population SE. Asterisks represent statistically differences between treated and untreated levels.

Effect of 3-AT on the uptake and accumulation of cadmium and lead by HFW cells

Increased metal uptake and accumulation in cells may enhance metal genotoxicity. Therefore, cellular cadmium levels were determined by atomic absorption spectrophotometry immediately (uptake) and 8 h (accumulation) after exposing cells to cadmium and/or 3-AT. Figure 4 shows that cadmium uptake (1–2.5 μM) in HFW fibroblasts exhibited dose-dependent increases ($P < 0.05$); the cadmium level remained the same 8 h after removal of this metal from the medium. Two-way analysis of variance showed that 3-AT co-treatment significantly enhanced cadmium uptake and accumulation in HFW fibroblasts when the cells were exposed to 1–2.5 μM of cadmium acetate; however, the enhancing effect of 3-AT was not observed at 10 μM of cadmium-treatment (Figure 4). Again, the cadmium levels in cells co-treated with cadmium plus 3-AT were constant when the cells were harvested immediately and 8 h after removal of the chemicals.

Figure 5 shows that lead uptake in HFW fibroblasts increased as lead acetate concentrations were increased ($P < 0.05$). Two-way analysis of variance showed that the lead levels in cells harvested 8 h after lead acetate treatment were not significantly different from those obtained immediately after treatment. 3-AT co-treatment significantly enhanced lead uptake and accumulation in HFW fibroblasts (Figure 5). However, 3-AT co-treatment did not affect lead cytotoxicity (Figure 2). These results show that lead cytotoxicity did not increase in proportion to cellular lead concentration.

Discussion

The present study has shown for the first time that cadmium and lead induce diploid human fibroblasts to form colonies in

Table I. Mutagenicity of HFW cells treated with cadmium and cadmium plus 3AT

Treatment[a]	No. of individual treatments	Replating efficiency[b]	Total clonable cells assayed ($\times 10^6$)	6-TG^r mutant frequency/10^6 clonable cells
Cd alone (μM)				
0	28	50.6 ± 4.0	14.17	6.84
2.5	9	62.8 ± 5.3	5.65	5.81
5	10	62.3 ± 8.2	6.23	5.22
10	9	58.0 ± 8.4	5.22	5.83
20	13	50.4 ± 3.6	6.55	9.96
40	13	54.0 ± 5.5	7.03	5.60
60	10	49.9 ± 3.5	4.99	5.30
3-AT + Cd (μM)				
0	17	55.1 ± 5.2	9.37	4.40
2.5	14	55.6 ± 4.9	7.78	7.08
5	14	41.8 ± 5.0	5.85	7.38
10	10	35.6 ± 8.6	3.56	8.47
MNNG (μM)				
0 (DMSO)	4	64.2 ± 3.7	2.57	14.63
10	4	65.6 ± 2.4	2.62	91.47[c]
12	4	53.3 ± 2.2	2.13	97.29[c]

[a]Cells were treated or not treated with 80 mM 3AT for 1 h, and then exposed to Cd for 24 h.
[b]Replating efficiency of cells at the time of mutant selection; mean ± SE.
[c]$P < 0.05$, Poisson distribution test.

Table II. Mutagenicity of HFW cells treated with lead and lead plus 3AT

Treatment[a]	No. of individual treatments	Replating efficiency[b]	Total clonable cells assayed (X 10^6)	6-TG^r mutant frequency/10^6 clonable cells
Pb alone (mM)				
0	28	50.6 ± 4.0	14.17	6.84
0.5	9	56.9 ± 5.7	5.12	6.04
1.5	8	54.4 ± 5.1	4.35	4.82
2	9	44.9 ± 4.1	4.04	6.81
3-AT + Pb (mM)				
0	17	55.1 ± 5.2	9.37	4.40
0.5	6	56.3 ± 4.4	3.38	7.04
1.5	6	52.0 ± 3.9	3.12	4.36
2	7	39.3 ± 3.0	2.75	7.33

[a]Cells were treated or not treated with 80 mM 3AT for 1 h, and exposed to Pb for 24 h.
[b]Replating efficiency of cells at the time of mutant selection; mean ± SE.

semi-solid media, i.e. anchorage-independent colonies. The anchorage-independent colonies were shown to be dose-dependently induced by these two metal compounds. The frequencies of anchorage-independent colonies induced by cadmium and lead are similar to those induced by the strong carcinogen MNNG at roughly equal cytotoxic dose ranges (30–10% survival). Anchorage independence is believed to be one step in the multi-step process of neoplastic transformation of human fibroblasts, and it has proven to be a particularly attractive endpoint for transformation studies, although the biochemical changes in anchorage-independent phenotypes are not yet fully understood (47–49). Several studies have suggested that anchorage independence results from a mutational event, due to in part the acquired anchorage independent phenotype is a permanent characteristic of the cells, and the dose-response curve for anchorage independence being similar to that for mutational markers (47–49). Moreover, anchorage-independent growth is related to modifications in signal-transduction pathways, e.g. it can be induced by growing cells in media containing high concentrations of serum or platelet-derived growth factor, and by expression of *sis* or *ras* oncogenes (47,48). Additionally, extopic expression of the transforming growth factor α-antisense gene in human cancer cells reduces the formation of anchorage-independent colonies and cell growth (56,57). Thus, anchorage independence may be generated through both epigenetic and genetic alterations. The finding that cadmium and lead induce anchorage independence, but not *hprt* gene mutations in diploid human fibroblasts suggests that these two metals act as human carcinogens by altering signal-transduction pathways.

A body of evidence shows that cadmium and lead stimulate signal-transduction molecules. Exposure of mammalian cells to cadmium chloride increases inositol triphosphate, releases Ca^{2+}, activates protein kinase C and triggers hormone-like responses through orphan-receptor signal pathways (37,38). Lead activates protein kinase C isolated from rat brains and stimulates the phosphorylation of membrane cytoskeletal proteins along a protein kinase C-dependent pathway, implying lead may alter cellular signal pathways (39,40). The ability of these two metals to induce anchorage independence is consistent with their ability to alter signal transduction in cells.

Cadmium and lead although do not increase mutation

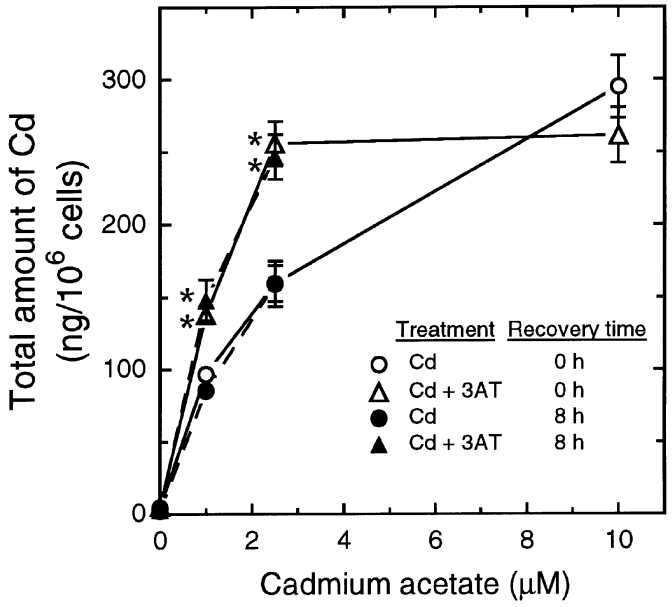

Fig. 4. Effects of 3-AT on Cd uptake and accumulation in human diploid fibroblasts. Cells in exponential growth were treated or not treated with 80 mM of 3-AT for 1 h. The cells were then treated or not treated with cadmium acetate for 24 h. Cells were then washed with PBS and one set of cells was cultured in complete medium for another 8 h. Cd concentrations in cells immediately after treatment (uptake; open symbols) or 8 h after removal of drugs from media (accumulation; closed symbols) were assayed as described in 'Materials and methods'. The results were obtained by averaging 3–6 independent treatments, and the bars denote population SE. Asterisks represent statistically differences between data obtained from cadmium alone and 3-AT plus cadmium at the same recovery time.

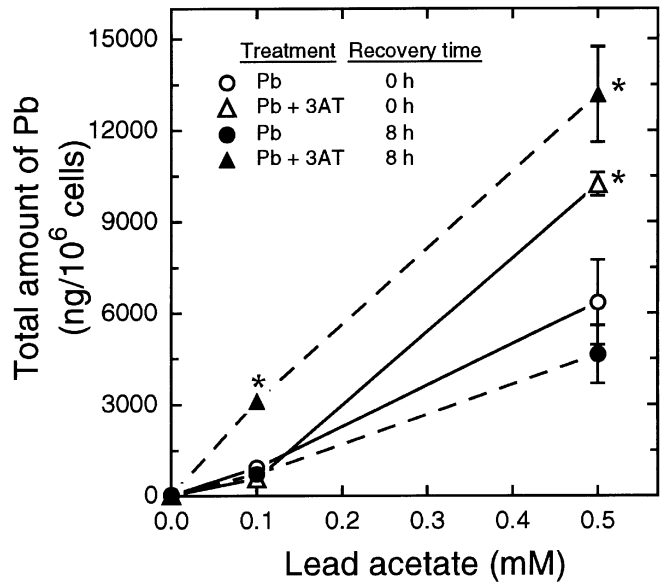

Fig. 5. Effects of 3-AT on Pb uptake and accumulation by human diploid fibroblasts. Cells in exponential growth were treated or not treated with 80 mM of 3-AT for 1 h. The cells were then treated or not treated with lead acetate for 24 h. Cells were then washed with PBS and one set of cells was cultured in complete medium for another 8 h. Pb concentrations in cells immediately after treatment (uptake; open symbols) or 8 h after removal of drugs from media (accumulation; closed symbols) were assayed as described in 'Materials and methods'. The results were obtained by averaging of 4–8 independent treatments, and the bars denote population SE. Asterisks represent statistically differences between data obtained from lead alone and 3-AT plus lead at the same recovery time.

frequency at the *hprt* gene in diploid human fibroblasts HFW, at similar cytotoxic dosages (~D_0), they can induce mutations at the same gene in CHO-K1 cells (8,21). That cadmium and lead mutagenicities observed in CHO-K1, but not in human fibroblasts may be attributable to that the genotoxic defence mechanisms against these two metals in HFW fibroblasts may differ from those in CHO-K1 cells. For example, the basal metallothionein level is ~5 μg per mg of protein in HFW (58), but undetectable in CHO-K1 (data not shown), and the catalase level is ~50-fold higher in HFW than in CHO-K1 (8,59, this study). Chinese hamster V79 cells, having functional metallothionein gene, also show weak mutagenicities by these two metals (60,61). This is consistent with the notion that metallothionein is the most important metal-binding protein, and accounts for resistance to metal genotoxicity (32). This comparison also suggests that metal mutagenicity is positively correlated to the cellular oxidative stress, i.e. mutation may be induced in cells having limited antioxidant machinery. As cadmium and lead, carcinogenic metals Ni^{2+} and As^{3+} have been reported to cause dose-dependent induction of anchorage independence, but not to induce mutations in diploid human fibroblasts (49).

3-AT significantly enhances cadmium-induced anchorage independence and cytotoxicity in diploid human fibroblasts HFW. The 3-AT's enhancing effect on cadmium genotoxicity is consistent with our previous observation that 3-AT enhances cadmium-induced cytotoxicity and mutagenicity in CHO-K1 cells (8), further supporting the notion that ROS is involved in the cadmium genotoxicity. Cadmium has been reported to induce gene expression of antioxidant enzymes, such as metallothionein and heme oxygenase (32,33), as well as H_2O_2 levels in cells (45). Cadmium may thus induce cellular antioxidant machinery to protect from its genotoxicity, as well as cellular ROS to cause genotoxicity. The intracellular catalase activity is markedly inhibited in 3-AT- and 3-AT plus cadmium-treated cells. Besides catalase inactivation ability, 3-AT has been reported to inhibit peroxidase (50), cytochrome p450IIE1 (51) and heme biosynthesis (52) that are also important to cope with cellular oxidative stress. Inactivating antioxidant machinery by 3-AT may result in increased levels of ROS in cadmium-treated cells, and subsequently enhanced cadmium genotoxicity because ROS can induce genotoxicity and signal-transduction pathways (41,42,62). The enhancing effect of 3-AT on cadmium-induced genotoxicity at low cadmium-treatment doses (1–2.5 μM) may be partly due to a higher cellular cadmium concentration enhanced by 3-AT; however, such enhanced cadmium uptake by 3-AT does not occur at high cadmium-treatment doses (10 μM). By contrast, 3-AT neither affects lead-induced anchorage independence and cytotoxicity, nor induces the weak mutagenicity of lead; no matter that 3-AT does enhance lead uptake and accumulation in HFW cells. This result suggests that catalase-associated antioxidant machinery is more important for cadmium than lead detoxification.

That enriched metallothionein in NIH/3T3 cells by transfection results in markedly decreased cadmium uptake (63) indicates that cellular antioxidants may affect metal uptake. Cadmium uptake by cells has been reported through calcium channels (64), anion exchangers (65), and membrane cadmium-binding proteins (66). 3-AT co-administration increases metal uptake may be due to in part its catalase inactivating function. Alternatively, 3-AT co-administration may decrease the amounts of metallothionein or other antioxidant enzymes, alter

membrane transporter systems or metal-binding proteins that would subsequently increase metal uptake. Additionally, the increased cellular lead levels by 3-AT co-administration may be attributable to formation of complexes with lead-binding proteins that can neither enhance transformation nor cytotoxicity.

Several kinds of *in vitro* transformation assays, including anchorage independence, morphological transformation, focus formation and immortalization have been applied to investigate chemical carcinogenesis. Cadmium and lead have been shown to cause morphological transformation or focus formation in Syrian hamster and Balb/3T3 cells (5,14,15). Focus formation and immortalization are rather difficult to be induced by carcinogens in diploid human fibroblasts (47–49,67). This is possibly due to the fact that diploid human fibroblasts exhibit limited lifespan, but cultured rodent cells do not. Induction of anchorage independence or focus formation is not sufficient to convert normal human fibroblasts into fully malignant cells (47,48,67). Exposure of immortalized human cells to a single carcinogen treatment or oncogene transfection, followed by selection for focus formation and anchorage-independent growth, yielded malignant tumours in athymic mice (67). Such a model system will be of help to elucidate metal carcinogenicity in human cells. Nevertheless, this present study has demonstrated that anchorage independence can be used as an early marker to detect phenotypic alterations in diploid human fibroblasts induced by carcinogenic metals; particularly, all the metal-exposed cells keep growth in our experimental conditions implying that anchorage independence induced by these metals may be an important factor in multi-step carcinogenesis.

In conclusion, this study clearly demonstrated that cadmium and lead induce anchorage-independent growth, but do not cause *hprt* gene mutations in diploid human fibroblasts, suggesting these two metals are human tumour-promoting agents. Catalase-associated antioxidant machinery may be involved in protection from anchorage-independent growth in cadmium-treated, but not in lead-treated cells. Cadmium is apparently stronger than lead and MNNG in inducing anchorage independence in human cells.

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