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Osteotoxicity of cadmium and lead in HOS TE 85 and ROS 17/2.8 cells: relation to metallothionein induction and mitochondrial binding

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Epidemiological, experimental and clinical data indicate that cadmium and lead are osteotoxins in man and other species. The relative sensitivities of a clonal human osteosarcoma cell line (HOS TE 85) and a clonal rat osteosarcoma cell line (ROS 17.28) to the cytotoxic effects of cadmium and lead were tested in serum-free media without added growth factors. The rat osteosarcoma cells were more sensitive to cadmium with cytotoxicity and inhibition of proliferation at 0.25 versus 0.75 and 1.0 μmol l^{-1} cadmium, respectively, for human osteosarcoma cell lines. The lower sensitivity to cadmium of human osteosarcoma cells is attributed, at least partly, to induction of metallothionein synthesis by cadmium and zinc in this cell line; in the rat osteosarcoma cell line, they do not induce metallothionein synthesis. Human osteosarcoma cells were more sensitive than rat osteosarcoma cells to lead with inhibition (IC_{50}) of proliferation at 4 μmol l^{-1} lead and cytotoxicity at 20 versus 6 and over 20 μmol l^{-1} lead, respectively, for these variables in rat osteosarcoma cells. Both cells lines attained the highest lead concentration in the 15 000 $\times$ g (mitochondrial) fraction. The lead in the mitochondrial, microsomal, nuclear and cytosolic fractions of the human cell line did not decrease during 24 h post-washout. Binding of lead was much less stable in the less sensitive rat cells, with 50–100% loss of mitochondrial, microsomal and nuclear lead during 24 h post-washout.

Keywords: cadmium, human osteosarcoma cells (HOS TE 85 cells), lead, osteosarcoma cells, rat osteosarcoma cells (ROS 17/2.8 cells)

Introduction

There is increasing evidence that both cadmium and lead are directly osteotoxic, independent of the renal tubular toxicity responsible for inhibition of synthesis of 1α,25 dihydroxy vitamin D_3 (D_3) and defective tubular reabsorption of phosphate.

In humans, cadmium pollution is epidemiologically associated with osteopenia and osteomalacia in both men and women without overt renal toxicity (Kido *et al.* 1989). It is considered causative in the severe osteomalacia diagnosed in endemic areas despite maximal bone cadmium concentrations below 2.0 μg g^{-1} (<19 μmol) in fatal cases of Itai-Itai disease (Nogawa *et al.* 1975). Cadmium produces bone lesions in vitamin D deficient weanling rats (Yoshiki *et al.* 1975), and is toxic to cultures of whole bone and bone cell lines (Miyahara *et al.* 1986, Kaji, *et al.* 1988, Angle *et al.* 1990a,b).

At current levels of lead exposure, human bone lead accumulates at approximately 0.1–0.25 μg g^{-1} per year of age. Excessive human lead exposure results in high bone lead concentrations (20–200 μg g^{-1} or 100–1000 μmol) with lead concentrated in the osteoclasts. Clinical osteotoxicity is manifest by impaired mineralization of osteoid, radiologically visible as 'lead lines' in childhood lead poisoning.

In osteoblast cultures, both cadmium and lead inhibit differentiation responses (Angle *et al.* 1990a,b, Long & Rosen 1992). In a clonal rat osteosarcoma cell line (ROS 17/2.8), the production of osteocalcin and cellular alkaline phosphatase activity are inhibited at below 1 μmol l^{-1} cadmium and at 1–5 μmol l^{-1} lead. The toxic effects can be prevented by the addition of D_3 to the culture media (Angle *et al.* 1990a,b, Thomas *et al.* 1990). The proliferative responses of ROS 17/2.8 osteoblasts

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are also inhibited at extremely low concentrations ($\leq 0.5\ \mu mol\,l^{-1}$) of cadmium but are not prevented by D_3 (Angle *et al.* 1990a,b). The sensitivity of ROS 17/2.8 cells to cadmium is at least in part due to the failure of cadmium to induce the synthesis of metallothionein in this cell line (Thomas *et al.* 1990, 1991).

The effects of cadmium and lead treatment upon proliferation and cytotoxicity in ROS 17/2.8 cells and in a human osteosarcoma cell line (HOS TE 85) are compared in this report to determine if the effects relate to the inducibility of metallothionein by cadmium or to the uptake and subcellular sequestration of lead.

Materials and methods

Materials

Cloned ROS 17/2.8 rat osteoblastic osteosarcoma cells (Rodan & Rodan 1984), supplied by Dr Gideon R. Rodan (Merck, Sharp & Dohme, West Point, PA) and HOS TE 85 human osteosarcoma cells (ATCC, Rockville, MD) were cultured in minimal essential medium (MEM) (Irvine Scientific, Santa Ana, CA) with $2\ mmol\,l^{-1}$ (or $2\ \mu mol\ ml^{-1}$) glutamine, and $100\ U\,ml^{-1}$ of penicillin and $100\ \mu g\,ml^{-1}$ streptomycin, supplemented with 10% fetal bovine serum (FBS) (Irvine), hereafter termed complete medium, at 37 °C in atmosphere containing 5% CO_2. For routine maintenance, media were changed every 3 or 4 days and cells subcultured weekly. For test procedures, cells were seeded at a density of $5000\ cells\,ml^{-1}$ culture medium in 35 mm diameter six-well plates. The media were changed twice at 24 h intervals and after 48 h the media were replaced with serum-free MEM containing the test substances.

Culture treatments

Distilled, deionized millipore-filtered water that contained no detectable traces of cadmium, zinc or lead was used to prepare all test solutions. To prevent precipitation of lead in the culture medium, lead acetate was diluted in deionized distilled water at $5\ mmol\,l^{-1}$. This stock solution was sterile filtered and diluted in water to 25 $\mu mol\,l^{-1}$ to $3\ mmol\,l^{-1}$ for addition to the media at 1:50 to obtain the final concentrations of 0.5 to 60 $\mu mol\,l^{-1}$. Cadmium and zinc chlorides did not require aqueous dilution before dilution in the media. The cadmium, lead and zinc concentrations of the media were confirmed by atomic absorption spectrometry.

Proliferative responses

The effects of cadmium, lead and zinc on DNA synthesis in cells were assessed by the method of Puzas & Brand (1986). Briefly, 5×10^4 cells were seeded in each well of 24-well plates and cultured in complete medium. After 24 h, the medium was replaced with complete medium without FBS plus cadmium, zinc or lead. After 24 or 48 h, thymidine at $5\ \mu mol\,l^{-1}$ (Sigma, St Louis, MO) and [^{3}H]thymidine at 1 μCi per well (New England Nuclear, Boston, MA) were added. Following a 2 h incubation at 37 °C, cells were harvested and processed for radioassay of ^{3}H uptake. Total DNA was measured by the method of Labarca & Paigan (1980). All assays were done on four wells at each concentration.

Studies of cellular viability used the procedure of Page *et al.* (1988). Aliquots containing 10^4 cells were seeded in each well of a 96-well microtiter plate (Corning, Corning, NY) and cultured for 24 h in complete medium. At 24 h after seeding, the medium was replaced with serum-free medium that contained various concentrations of cadmium, lead and zinc. After 24 or 48 h, this medium was removed, cell layers washed once with complete medium and replaced with complete medium without phenol red containing $0.5\ mg\,ml^{-1}$ of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma). After a 2 h incubation at 37 °C, the MTT-containing media were removed and cell layers containing the reduced formazan metabolite of MTT were solubilized on 0.2 ml dimethylsulfoxide (Sigma). Absorbances of solubilized cell layers were determined at 540 nm using a V_{max} kinetic microplate reader (Molecular Devices, Menlo Park, CA). All assays were done on eight wells, each concentration.

Metallothionein synthesis

Omission of FBS from the medium removes uncharacterized serum factors that induce metallothionein gene expression in cultured cells (Imbra & Karin 1987). L-[^{35}S]cysteine ($10\ \mu Ci\,ml^{-1} = 8.3\ \mu mol\,l^{-1}$ cysteine) (New England Nuclear) was added to cysteine-free MEM (MEM Select-Amine Kit, Gibco, Gaithersburg, MD) to label newly synthesized metallothionein molecules. After 24 h, cells were harvested by trypsinization, collected by centrifugation at $13\,000 \times g$ for 10 min at room temperature and washed once with ice-cold Hank's balanced salt solution.

Cell pellets were suspended in $10\ mmol\,l^{-1}$ Tris, pH 7.5 and ultrasonicated (Micro Ultrasonic Cell Disrupter, Kontes, Vineland, NJ) and the resulting lysates centrifuged at $13\,000 \times g$ for 5 min at room temperature to prepare cystosolic fractions for chromatography. Aliquots of cystosol were applied to a Sephadex G-75 gel (Sigma) column with bed dimensions of 1.5×45 cm which was eluted with $10\ mmol\,l^{-1}$ Tris, pH 7.5. Eluate fractions (1 ml) were collected and a portion of each fraction prepared for ^{35}S radioassay in a model LS5801 liquid scintillation spectrophotometer (Beckman, Houston, TX).

Lead uptake and retention

For lead uptake, 10^7 cells per flask were incubated with $10\ \mu mol\,l^{-1}$ lead in serum-free media for 24 h. At the specified intervals, duplicate cell layers were washed three times with PBS, trypsinized and cell pellets frozen. At 24 h

duplicate sets of cells were washed and recultured in serum-free, lead-free media. Cells were washed three times with PBS, and cell pellets frozen at 1, 2, 4, 8 and 24 h. All cell pellets were sonicated in 1.0 ml H_2O for lead determination at the same time.

Subcellular fractions were obtained by homogenizing cell pellets in 0.25 mol l^{-1} sucrose and using centrifugation at $600 \times g$ for 10 min (nuclei), $15000 \times g$ for 10 min (mitochondrial and liposomes) and $100000 \times g$ for 60 min (plasma membrane and microsomes). The $100000 \times g$ supernate (cytosol) was also analyzed.

Lead concentrations were determined as the means of two samples, each in duplicate, by atomic absorption with a graphite furnace (Model 2380, Perkin Elmer, Norwalk, CT), using a L'VOV platform. Lead standards (2–30 ng ml^{-1}) were diluted from Atomic Spectral Standard (J. T. Baker, Phillipsburg, NJ). Quality control standard materials were from NIST, Gaithersburg, MD. Samples were diluted 10- to 500-fold in 1% nitric acid to express results within the linear range of standards.

Statistical analysis

All concentrations are plotted as the mean ± SD. The 50% inhibition concentrations (IC_{50}) were determined by log-probit analysis. Changes in cell viability and cellular uptake were first subjected to arc-sine transformation recommended for percentages (Kleinbaum & Kupper 1978) followed by analysis of variance testing using Number Cruncher Statistical System, version 5.01 (NCSS, Kaysville, UT). Paired data were then analyzed by Student's *t*-test, with significant differences considered as $P < 0.05$.

Results

Cadmium inhibited the 24 h incorporation (IC_{50}) of [^{3}H]thymidine in ROS 17/2.8 at 0.25 μmol l^{-1} and at approximately 1.0 μmol l^{-1} in HOS TE 85 ($P < 0.05$). Cytotoxicity (MTT IC_{50}) was evoked by 0.25 μmol l^{-1} cadmium in ROS 17/2.8 and by 0.75 μmol l^{-1} cadmium in HOS TE 85 ($P < 0.05$) (Figure 1).

The dose response to lead was initially evaluated at 48 h. Proliferation as assayed by the uptake of [^{3}H]thymidine was inhibited (IC_{50}) in HOS TE 85 by 4.2 μmol l^{-1} lead versus 6.7 μmol l^{-1} in ROS 17/2.8 ($P < 0.05$). Cytotoxicity (IC_{50}) was evident at 20 μmol l^{-1} lead in HOS TE 85 but at over 50 μmol lead in ROS 17/2.8 (Figure 2).

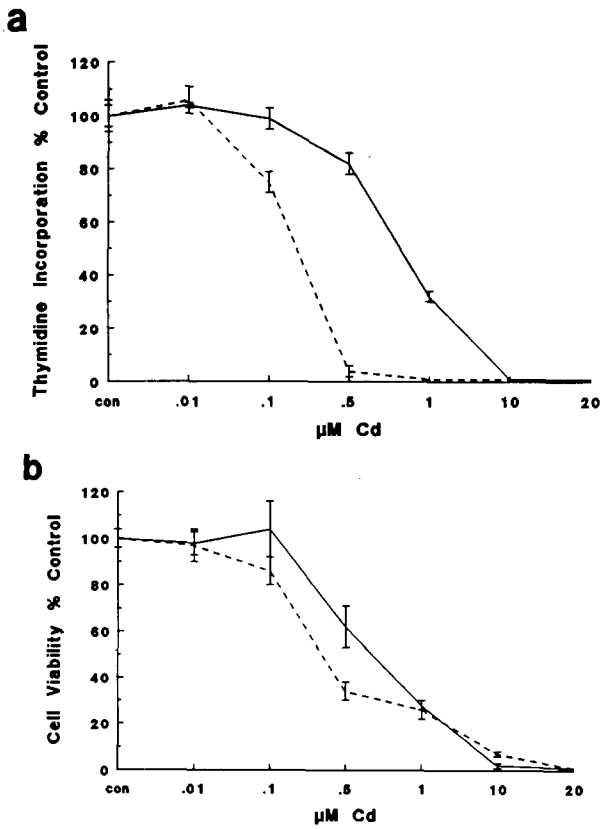

Figure 1. Response as percent of control to 24 h incubation with cadmium chloride (μmol l^{-1}) of (**a**) [^{3}H]thymidine incorporation and (**b**) cell viability (MTT assay) of HOS TE 85 (——) and ROS 17/2.8 (– – –) cells.

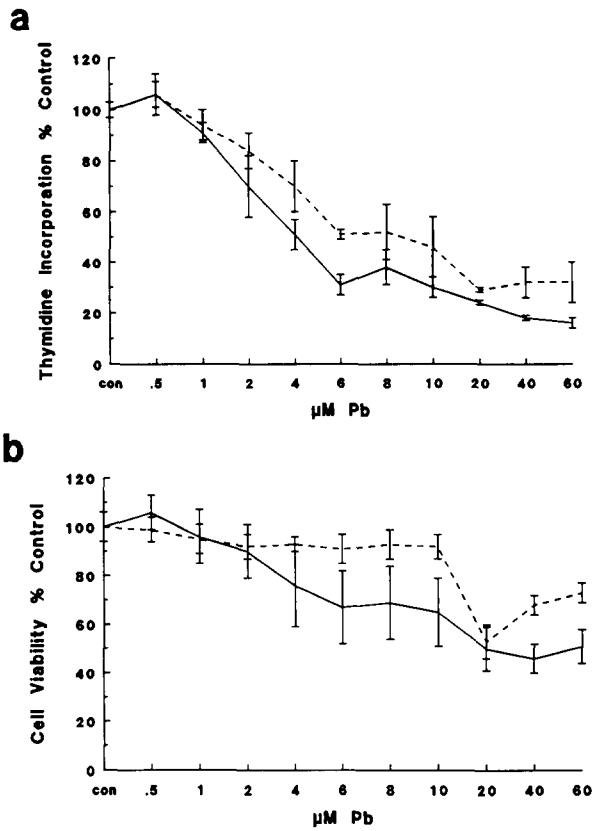

Figure 2. Response as percent of control to 48 h incubation with lead acetate (μmol l^{-1}) of (**a**) [^{3}H]thymidine incorporation and (**b**) cell viability (MTT assay) of HOS TE 85 (——) and ROS 17/2.8 (– – –) cells.

Thymidine uptake was inhibited (IC_{50}) in both cell lines by 24 h incubation with 25–50 μmol l^{-1} zinc. Cytotoxicity (IC_{50}) was found in both cell lines at greater than 50 μmol l^{-1} zinc for 24 h. In contrast to the cadmium responses, there was not a significantly greater sensitivity to zinc by the HOS TE 85 (Figure 3).

Metallothionein was not identified in fractions 45–60 from ROS 17/2.8 cells treated with 25 μmol l^{-1} zinc and 0.5 μmol l^{-1} cadmium but was identified in HOS TE 85 cells (Figure 4).

Washout experiments tested the persistence of inhibition. At 24 h incubation with lead there was 50% inhibition of [^{3}H]thymidine uptake and cell viability of both cell lines at 4–5 μmol l^{-1} lead. A dose-related response to prior exposure to lead was still evident in both cell lines 24 h after replacement with lead-free, serum-free media (Figure 5).

The lead content per cell layer was comparable in HOS TE 85 and ROS 17/2.8 during 24 h incubation with 10 μmol l^{-1} lead but continued to increase in HOS TE 85 post-washout ($P < 0.05$ at 2–24 h) (Figure 6). HOS TE 85 grow more rapidly and contain over twice the DNA per cell layer of ROS 17/2.8 at 24 h. The rapid decline in the amount of lead (μg) per gram of DNA of ROS 17/2.8 post-washout (Figure 6) despite a constant level of DNA per cell layer suggested less stable binding of lead to DNA in association with decreased sensitivity to lead. This was tested by direct assay of the subcellular lead concentrations before and after washout.

Differential centrifugation of the cell pellets after 24 h incubation with 10 μmol l^{-1} lead and at 24 h post saline washout demonstrated a rapid decrease of the amount of lead (ng) per milligram of protein of the ROS 17/2.8 in the nuclear ($600 \times g$), mitochondrial ($15\,000 \times g$) and microsomal fractions ($100\,000 \times g$) (Figure 7). In contrast, there was no loss of lead from any of the subcellular fractions or the cytosol of HOS TE 85 after 24 h incubation with

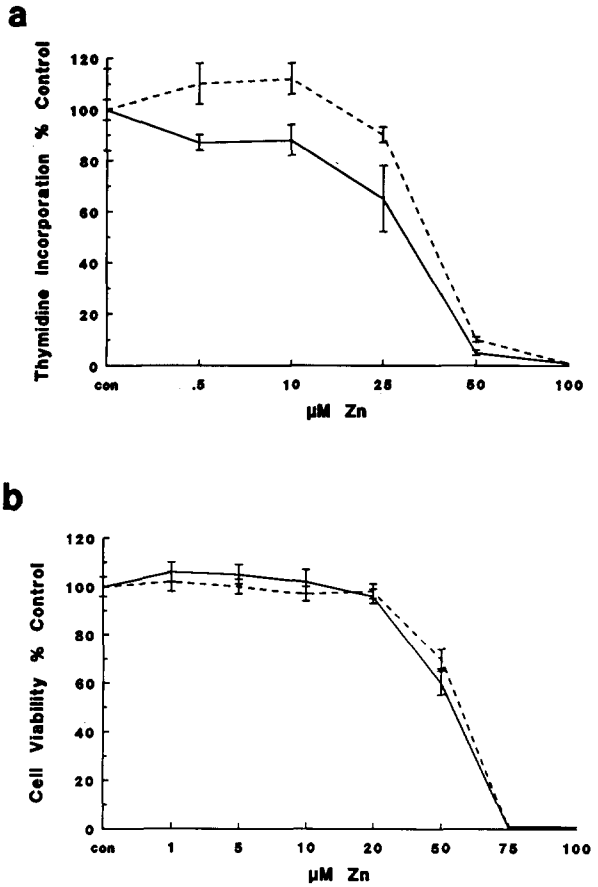

Figure 3. Response as percent of control to 24 h incubation with zinc chloride (μmol l^{-1}) of (**a**) [^{3}H]thymidine uptake and (**b**) cell viability by HOS TE 85 (——) and ROS 17/2.8 (– – –) cells.

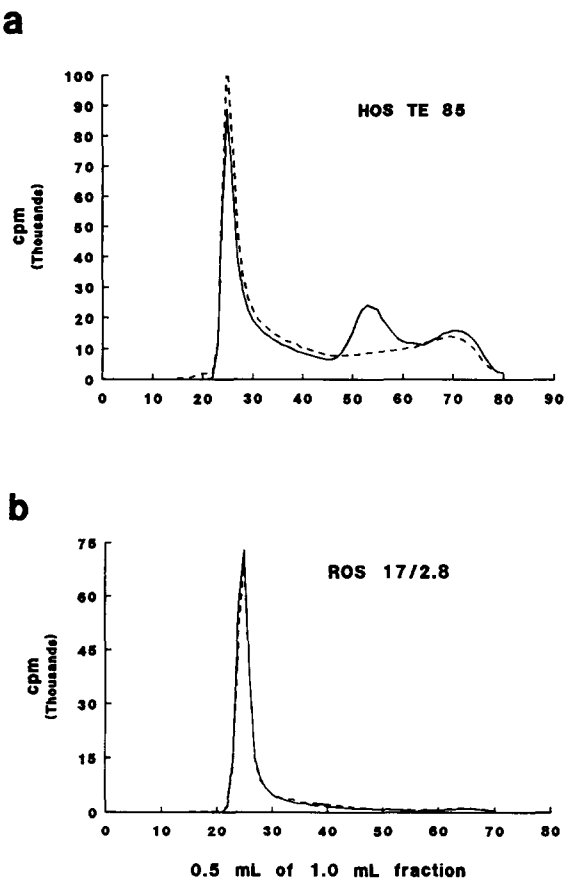

Figure 4. Sephadex G-75 gel chromatographic profiles of ^{35}S elution for cytosols prepared from (**a**) HOS TE cells and (**b**) ROS 17/2.8 after 24 h incubation with 0.5 μmol l^{-1} cadmium (——) and without cadmium (– – –).

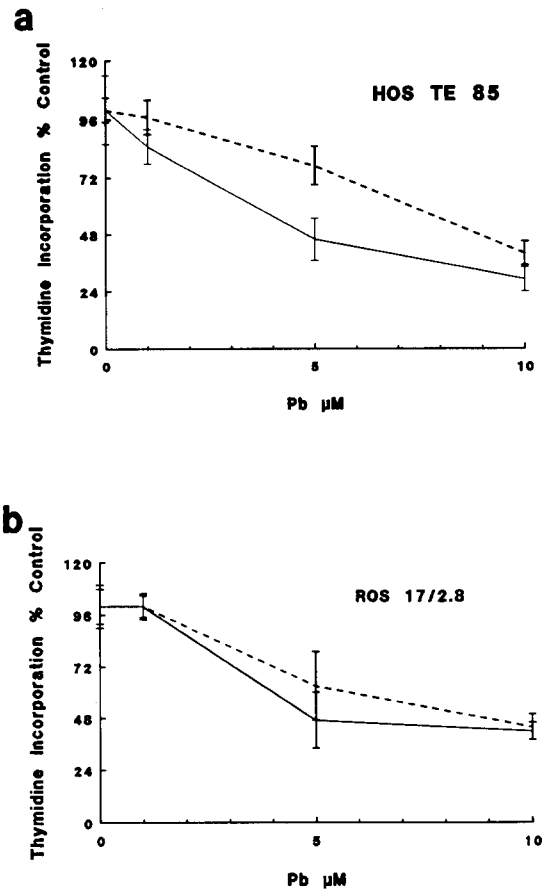

Figure 5. Response as percent of control of [^{3}H]thymidine incorporation by (**a**) HOS TE 85 and (**b**) ROS 17/2.8 at the end of 24 h incubation (——) with 1.5 and 10 μmol l^{-1} lead nitrate, and after an additional 24 h in lead-free, serum-free media (post-wash) (- - -).

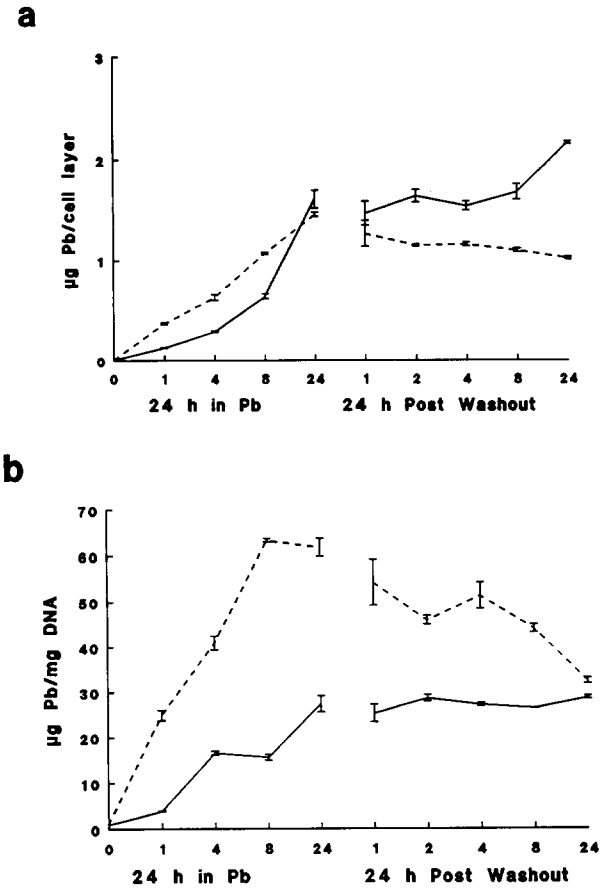

Figure 6. Lead content (**a**) μg g^{-1} cell layer and (**b**) μg g^{-1} DNA of HOS TE 85 (——) and ROS 17/2.8 (- - -) cells during 24 h incubation in serum-free media with 10 μmol l^{-1} lead and during the subsequent 24 h (washout) in lead-free, serum-free media.

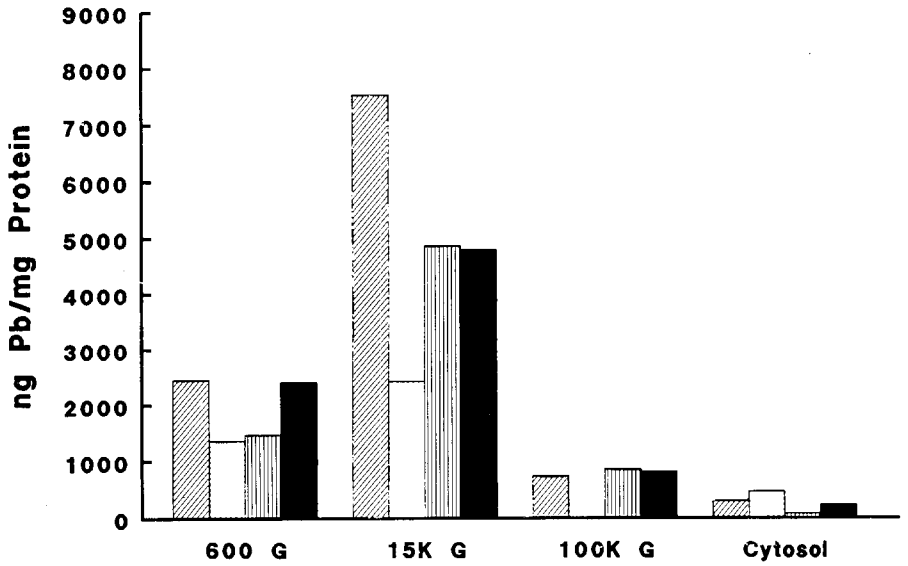

Figure 7. Subcellular lead [ng mg^{-1} protein (mean of two samples)] of ROS 17/2.8 and HOS TE 85 at 24 h incubation with 10 μmol l^{-1} lead and 24 h post saline wash in lead-free, serum-free media. ▨, ROS; □, ROS/post; ▥, HOS; ■, HOS/post.

saline. In both cell lines the maximal concentration of lead was in the 15000 × *g* (mitochondrial) fraction.

Discussion

The extreme sensitivity to cadmium of two species-distinct osteosarcoma cell lines is consistent with human, animal, bone culture and bone cell culture data of direct osteotoxicity at less than 1 μM cadmium. ROS 17/2.8, which are even more sensitive to cadmium than HOS TE 85, have an altered modulation of metallothionein. No metallothionein synthesis is induced by either cadmium or zinc although gene expression is enhanced by azacytidine and sodium butryate (Thomas *et al.* 1990, 1991). Deficiencies of other factors may also be altered since ROS 17/2.8 were not more sensitive to zinc than HOS TE 85. The extreme sensitivity of these osteosarcoma cells to cadmium may merit investigation of the chemotherapeutic potential of cadmium compounds.

The washout experiments were conducted to determine if the greater tolerance for lead of ROS 17/2.8 related to differences in uptake or subcellular sequestration. The uptake of lead per cell layer was comparable although the amount of lead (μg) per gram of DNA was higher in the less rapidly growing ROS 17/2.8. The greater retention of lead post-washout by HOS TE 85 suggests a tightly bound complex such as the lead phosphate protein site proposed by Long *et al.* (1990). The more persistent retention of lead in the slightly more lead-sensitive HOS TE 85 cells indicates that this mitochondrial binding is not necessarily a non-toxic sequestration of lead.

The inhibition of proliferative responses in clonal osteoblastic cell lines from two species provides additional evidence for direct osteotoxicity at micromolar concentrations of cadmium and lead.

Acknowledgments

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