Pattern of Stress Protein Expression in Human Lung Cell-Line A549 after Short- or Long-Term Exposure to Cadmium

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Exposure to cadmium is associated with the development of pulmonary damage such as emphysema and lung cancer. This metal is also a powerful inducer of stress proteins in many biologic models. The present study was undertaken to evaluate whether an overexpression of the heat shock protein (hsp)72 stress protein, which indicates repair of damaged proteins, could be a sensitive and early biomarker of environmental pollution by Cd. In comparative studies, we examined the effects of exposure to Cd (as $CdCl_2$) on the growth rate of the A549 pulmonary cell line, and (by Western blot analyses) on the induction of the hsp72 stress protein and metallothioneins (MTs). $CdCl_2$ exposure was studied for periods of 2 hr to 1 month. For short-term exposure (2–6 hr) to Cd concentrations higher than 50 μM, an overexpression of hsp72 appeared 6 hr later, suggesting that hsp72 might be considered an early biomarker of acute exposure to Cd. For exposures lasting more than 4 days, lower doses of Cd (0.1–10 μM) similar to levels encountered in occupational exposure induced a significant increase of the hsp72 level. Because the increase of hsp72 occurs for doses that did not affect cell proliferation, our work supports the idea that its overexpression might be used as a sensitive indicator of occupational exposure to Cd. However, increased resistance to Cd appeared in A549 cells exposed for 1 month and overexpression of hsp72 disappeared simultaneously. It is possible that, *in vivo*, cell adaptation also occurs throughout chronic exposure to Cd, with a decrease of hsp induction as a consequence. A dose-related increase of MTs was found after 4 days of exposure to Cd concentrations ranging from 0.1 to 10 μM without change of overexpression during chronic exposure, suggesting that MT expression could be a more constant indicator of Cd pollution. Because 0.1 μM Cd (11 μg/L) induces hsp72 expression, showing the presence of damaged proteins, our work suggests that the maximum allowable biologic exposure limit should be lowered. *Key words*: cadmium, chronic exposure, heat shock proteins, lung epithelial cells, metallothioneins. *Environ Health Perspect* 108:55–60 (2000). [Online 13 December 1999]

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Stress proteins, also known as heat shock proteins (hsp) because they were first observed in insect cells as a consequence of sublethal hyperthermia (*1*), accumulate in cells exposed to a variety of environmental aggressions (heat, alcohols, heavy metals, γ-rays, etc.) (*2–5*). This class of proteins plays many physiologic roles within a cell, from directing the folding of newly synthesized proteins to targeting damaged or useless proteins to degradation (*6–7*). Overexpression of stress proteins also confers a higher resistance to aggressions that otherwise could be lethal, leading to strategies of preconditioning of animal tissues before surgery with sublethal hyperthermia or hypoxia (*8,9*).

Stress proteins are an interesting field of research because their expression can be interpreted as an early and probably sensitive bioindicator of cell aggression and/or mild suffering and as a molecular strategy to increase resistance toward adverse situations.

The present work deals with the effect of cadmium on the A549 cell line, which is derived from human lung alveolar epithelium. Cadmium is a harmful pollutant that is widely present in both water and the atmosphere. It is inhaled during smoking as a trace component from cigarette smoke (10–20 μg Cd inhaled/cigarette) (*10*). As Cd mediates its toxic effects through the production of reactive oxygen species and the modulation of cellular GSH levels (*11*), resulting in accumulation of denatured proteins, it strongly promotes the expression of stress proteins in various biologic models (*12–14*).

From preliminary experiments based on densitometric analysis of autoradiographs of electrophoretically separated ^{35}S-methionine-labeled proteins, we observed that in A549 cells, the HSP70 family was the most overexpressed group of stress proteins under stressing conditions and especially after exposure to heavy metals. Therefore, our present study mainly deals with the inducible form of the HSP70 family—hsp72. Our aim was to determine the lowest concentration of Cd able to trigger the stress response in A549 cells, and to use this concentration to evaluate the possible use of the hsp72 overexpression as a biomarker of Cd exposure.

In addition to the effects of short-term exposures (from 1 hr to 4 days), we were also interested in investigating long-term cultures, carried out in the permanent presence of weak concentrations of Cd, to determine the duration of the stress reaction evidenced by a higher level of stress proteins as compared to controls. Cadmium accumulates in alveolar epithelial cells (*15*), hepatocytes, and kidney cells (*16*) after chronic exposure. It was therefore of interest to explore the possible increase in the toxicity of the metal and to evaluate the threshold concentrations that did not affect cell growth during long-term exposure.

Metallothioneins (MTs), another family of highly inducible proteins, may contribute to protection against Cd. MTs are small metal binding proteins that are strongly induced by Cd (*17*) and that have been proposed to protect the organism from metal toxicity (*18*). Therefore, we also examined the ability of Cd to trigger the expression of MTs under our experimental conditions.

Materials and Methods

Chemicals and reagents. Monoclonal mouse antibodies raised against hsp72 (SPA810), hsp72/73 (SPA 822, clone BB70) or hsp90 (SPA 830), were from StressGen Biotechnologies (Victoria, British Columbia, Canada). Monoclonal mouse antibody against MT (MT-1 and MT-2, clone E9) was obtained from Dako (Trappes, France). Peroxidase and fluorescein isothiocyanate (FITC) goat antimouse IgG antibodies were purchased from Sigma (Lyon, France). Protease inhibitors (aprotinin, leupeptin, pepstatin) were from Boehringer-Mannheim (Meylan, France). Fetal calf serum (FCS) was from D.A.P. (Volgegrun, France) and cell culture reagents were from Gibco-Life Technologies (Cergy Pontoise, France).

Cell culture. The A549 cell line has been established in permanent culture from a human lung adenocarcinoma (*19*). *In vitro*, these cells are largely differentiated as alveolar epithelial cells, type II (*20*). For routine culture, cells were propagated in Dulbecco modified Eagle medium (DMEM; glucose 4.5 g/L) supplemented with 10% FCS and nonessential amino acids, in the presence of

gentamycin (20 µg/mL) and grown at 37°C in a humidified 5% CO_2 atmosphere. For experiments, cells were seeded at a density of 2×10^4 cells/cm^2 in petri dishes (60 mm in diameter). Under such conditions, confluence was reached within 4–5 days. Cell proliferation was estimated by cell counting (Coulter counter, Coultronics, France). Cell viability was determined by trypan blue exclusion.

Experimental cultures for investigating the induction of stress proteins by Cd. Short-term exposures to Cd were achieved at day 4 of the cultures by adding $CdCl_2$ at concentrations ranging from 50 to 200 µM into the culture medium for 2, 4, 6, 24, and 48 hr, then changing the medium into fresh DMEM and placing the cells under standard conditions for 2–48 hr to discover the time-dependent increase of hsp72 expression.

For long-term exposures (4 days–1 month) to Cd, we cultured and subcultured the cells in the permanent presence of the metal at concentrations of 0.1, 1, or 10 µM. For the first set of cultures, Cd was added as soon as cells were attached, i.e., approximately 2 hr after seeding. Subcultures were carried out on day 4 or 5 of cultures: cells were counted in a suspension obtained by trypsin–EDTA treatment of both control and experimented cultures and then seeded at the same density (2×10^4/cm^2) in the presence of either standard DMEM (controls) or Cd-supplemented medium.

In all cases, three cultures were treated in parallel for each Cd concentration and three control cultures (not submitted to Cd treatment) were processed similarly for comparison.

Protein radiolabeling and gel electrophoresis analysis. Qualitative and quantitative estimation of the *de novo* synthesized proteins was carried out as previously described (*21*). Briefly, cells were incubated for 1 hr in methionine-free DMEM containing 2% FCS. For short-term exposures (< 24 hr), ^{35}S-methionine (15 µm µCi/mL) was added at the end of Cd exposure and incubated for 20 hr. A 20-hr lag period was chosen because previous results indicated that this delay was necessary to obtain a maximal expression of stress proteins in that cell line. In long-term exposures (> 24 hr) ^{35}S-methionine was present during the last 20 hr of exposure. The medium was then removed and the cell layer was rinsed with ice-cold phosphate buffered saline (PBS), scraped with a rubber policeman, and collected in lysis buffer (PBS containing 10 mM EDTA, 0.5% NP-40, 1 mM PMSF, 1 µg/mL aprotinin, 2 µg/mL leupeptin, and 2 µg/mL pepstatin, pH 7.4), and stored at -80°C. Before their use, cell homogenates were thawed and sonicated (10 sec), and their protein contents were determined as described by Lowry et al.

(*22*). Equal amounts of protein (20 µg) from cell lysates were separated by SDS-PAGE according to the method of Laemli (*23*). After the gel dried, labeled proteins were revealed by autoradiography.

Western blot analysis. Proteins separated by SDS-PAGE were electroblotted onto 0.45-µm nitrocellulose membranes as described by Towbin et al. (*24*). The quality of the transfer was verified by protein staining with Ponceau red. To saturate the nonspecific sites, the blots were incubated with a blocking buffer [tris-buffered saline (TBS): 10 mM Tris, 140 mM NaCl, pH 7.4] containing 0.3% Tween 20 (1 hr, 37°C). The blots were further incubated for 1 hr at room temperature (20°C) in the presence of monoclonal antibodies directed against hsp72, hsp72/73, or hsp90, diluted 1:20,000; 1:60,000; and 1:15,000; respectively, and then allowed to stand overnight at 4°C. After washing with TBS for 5 min, TBS containing 0.1% Nonidet 40 for 15 min, and TBS for 5 min (twice), the blots were incubated for 2 hr in the dark at room temperature in the presence of a peroxidase-conjugated antibody goat antimouse IgG diluted 1:20,000 in TBS containing 3% dried skim milk. After a second wash, the blots were incubated with the electrogenerated chemiluminescence (ECL) reagent as specified by the manufacturer (Amersham, Les Ulis, France) for 2 min at room temperature and then exposed to ECL hyperfilm (Amersham). HSP levels were determined by computer-assisted densitometric analysis of the autoradiographs.

The procedure was modified for the detection of MTs. Because MTs have a low molecular weight, SDS-PAGE was performed using 18% acrylamide gels. Because standard immunoblotting procedures do not allow the detection of MTs, we used the method developed by Mizzen et al. (*25*); 2 mM $CaCl_2$ was included in the electrophoretic transfer buffer and the nitrocellulose membranes were fixed in 2.5% glutaraldehyde for 1 hr. The membranes were then washed 3 times in PBS for 5 min and 50 mM monoethanolamine was added to the third wash solution to quench any residual glutaraldehyde.

Indirect immunofluorescence. Cells were seeded onto sterile glass coverslips (1 cm diameter) that were placed on culture wells and allowed to grow for 2–3 days. After exposure to Cd, cells on the coverslips were fixed in methanol for 10 min at -20°C, dried, and stored at -20°C. After rehydration in PBS, cells on coverslips were incubated for 1 hr at 37°C in the presence of mouse IgG raised against hsp72 (diluted 1:500 in PBS), washed, and incubated with antimouse IgG coupled to FITC (diluted 1:100). Nuclei were counterstained with propidium iodide

(0.1 µg/mL PBS for 5 min). The glass coverslips were mounted with Fluoprep (BioMérieux, Lyon, France) and were observed using an Olympus-BH2 ultraviolet microscope (SCOP-Olympus, Merignac, France). Photographs were taken with an Olympus OM4 camera using Ektachrome 320T film (Eastman Kodak, Rochester, NY).

Results

Stress protein synthesis as a response to short-term exposures to Cd. A549 cells were submitted to Cd concentrations ranging from 0.1 to 200 µM for 2, 4, 6, 24, or 48 hr, then incubated for increasing times in normal culture medium. An overexpression of hsp72 appeared 6 hr after Cd exposure; it reached its maximum 20 hr later (not shown). Consequently, hsp72 expression was always evaluated after 20 hr of recovery in Cd-free medium.

An autoradiograph of the ^{35}S-methionine-labeled proteins subjected to SDS-PAGE (Figure 1A) shows the profile of radiolabeled proteins obtained from cells grown for 24 hr in the presence of different Cd concentrations. The pattern of protein expression is similar in all of the cell extracts except for proteins of approximately 70 and 90 kDa; they are clearly over-expressed in cells exposed to 100 and 200 µM Cd.

The Western blot analysis (Figure 1B) of the same cellular extracts demonstrates the overexpression of hsp90 and hsp72 after exposure to Cd concentrations ranging from 25 to 200 µM. Densitometric analyses of hsp72 expression in cells exposed to different Cd concentrations for 1–48 hr are summarized in Figure 2. The ability of a given Cd
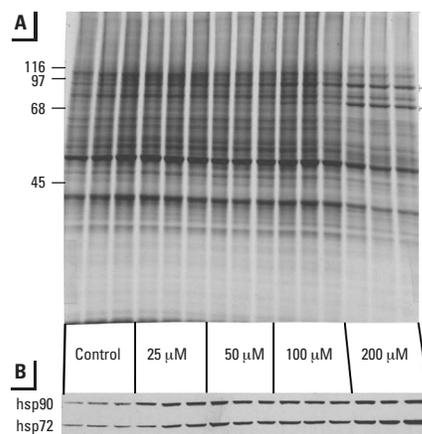

Figure 1. Analysis of hsp72 and hsp90 expression in A549 cells grown for 24 hr in the presence of different Cd concentrations. (*A*) Profile of *de novo* synthesized proteins during the 20 hr after Cd exposure. Twenty micrograms of ^{35}S-methionine-labeled proteins from each cell culture were separated on 10% polyacrylamide gel and autoradiographed. (*B*) Immunoblot characterization of hsp72 and hsp90.

concentration to trigger an overexpression of hsp72 seems to depend on the duration of exposure. For short-term exposures (2, 4, and 6 hr), a significant induction of hsp72 resulted from concentrations of 50 to 200 μM Cd and a maximal expression was observed at 200 μM. After a 24-hr exposure to Cd, overexpression of hsp72 occurred at 1 μM ($p \leq 0.05$) and was more important ($p \leq 01$) at 10 μM Cd. After a 48-hr exposure, a similar pattern of induction was found for Cd concentrations 10 times lower (from 0.1 to 10 μM). The highest doses (> 50 μM, 48 hr) did not permit de novo protein synthesis as seen after ^{35}S-methionine incubation, which explains the observed relative decrease of hsp72 expression.

hsp72 expression after long-term exposures to Cd. To determine whether an overexpression of hsp72 can serve as a biomarker of chronic exposure to Cd, A549 cells were cultured for 1 month in the presence of 0.1, 1, or 10 μM CdCl$_2$. These doses were chosen because they did not affect the proliferation rate of cells much and because they did not lead to cell death, as verified by trypan blue exclusion. Indeed, as seen in Figure 3, no significant change in cell growth was observed after 4 days of culture in the presence of 0.1 μM Cd. The doses of 1 and 10 μM slowed the proliferation rate, resulting in a respective 15 ± 5% and 50 ± 6% decrease of the cell number at day 4 of culture. Conversely, the highest dose (50 μM Cd) was incompatible with long-term cell growth.

At the end of each culture, control and Cd-exposed cells were dissociated, then seeded
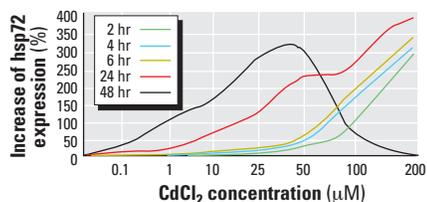
at the same density in either control or Cd-supplemented medium to carry out the next culture. Figure 4 shows the growth inhibition due to Cd through successive cultures. It appears that A549 cell susceptibility to Cd decreases with time. After 1 month of culture in the presence of the metal, no change of proliferation rate was observed with the highest dose (10 μM) as compared to controls.

Under the same experimental conditions, the expression of hsp72 was analyzed. As compared to controls, 10 μM Cd induced a 230% increase of hsp72 expression after 4 days of exposure (Figure 5). However, for longer periods of culture in the presence of the metal, this effect faded away until no response was seen at day 31. The same trend, although to a lower degree, was observed due to the chronic exposure of cells to 1 μM Cd. The level of hsp72 expression at day 4 (110% increase as compared to controls) decreased gradually until day 31. Exposure to concentrations as low as 0.1 μM Cd still increased hsp72 expression significantly ($p < 0.05$) in the first subcultures.

As seen in Figures 4 and 5, an inverse relationship appears between the magnitude of growth inhibition and the overexpression of hsp72.

The hsp72 overexpression was confirmed by using a monoclonal antibody directed against both inducible (hsp72) and constitutive (hsp73) heat shock proteins (Figure 6). It appears that the synthesis of hsp72 was highly

increased after 3 days of culture in the presence of 10 μM CdCl$_2$, whereas it was similar to that of controls after 31 days of culture. Conversely, the amount of hsp73 was almost constant in cells exposed to 10 μM Cd for 3 or 31 days, as compared to controls.

Immunofluorescence staining of hsp72. Detection of hsp72-containing cells was performed by indirect immunofluorescence staining (Figure 7). As compared to controls that were negative (Figure 7A), cells exposed to 100 μM Cd for 1 hr displayed a strong fluorescence in both cytoplasm and nucleus (Figure 7B). In contrast, chronic exposures to low doses of Cd (10 μM) yielded a strong fluorescence only in some cells (Figure 7D). Labeling with propidium iodide showed nonreactive cells in the same culture (Figure 7C). Therefore, the number of hsp72 immunoreactive cells versus nonimmunoreactive cells during chronic exposure to Cd could be evaluated (Figure 8). Highly fluorescent cells (5%; hsp positive) were obtained from 10 μM Cd at day 3 of the culture. The number of hsp72-positive cells decreased with time to reach approximately 0.2% at the culture day 31. In contrast, in cultures exposed to the lowest Cd doses (< 1 μM) a small number of hsp72-positive cells was found, which does not fit with the hsp72 overexpression observed on immunoblots. It is possible that in cultures exposed to low doses of Cd, numerous cells that were slightly fluorescent were not counted. Therefore, hsp72 overexpression, as seen on immunoblots, is more representative of the global induction of the hsp in the culture than immunofluorescent staining.

Long-term exposure to Cd induces resistance to the metal cytotoxicity. To understand the lack of hsp72 overexpression in cells exposed to 10 μM Cd for > 1 month (referred to as CD31), cytotoxicity (evaluated on cell proliferation) was investigated on these CD31 cells, either cultivated in the continuous presence of 10 μM Cd or replaced for 15 days in Cd-free medium (referred to as CD31–15). The 4-day exposure of the three strains to 10 and 25 μM Cd (Table 1) revealed a relative acquired resistance of CD31 and CD31–15 strains to the metal as compared to cells grown in Cd-free medium. The 10 μM Cd concentration, which induced a 50% decrease of A549 cell growth, did not change either CD31 or CD31–15 proliferation rates.

Figure 2. Variations of hsp72 overexpression in terms of CdCl$_2$ concentration and duration of exposure. Values correspond to the average of five separate experiments.
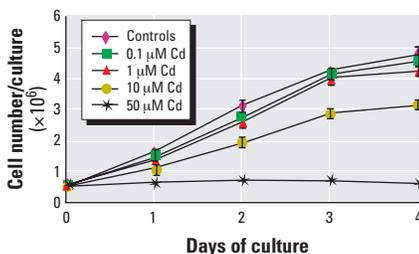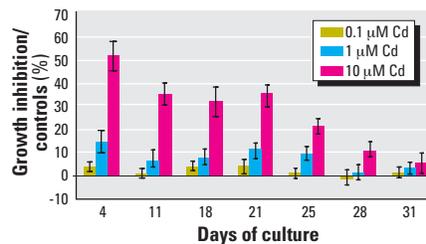

Figure 3. Growth rate of A549 cells cultured in standard (controls) or Cd-complemented culture medium. Cadmium was added 2 hr after seeding, when cells were attached. Values correspond to the mean of three cultures ± standard error.

Figure 4. Effects of chronic exposure to Cd on the proliferation rate of A549 cells. Histograms show the percent of growth inhibition at the end of each successive culture resulting from exposure to Cd.
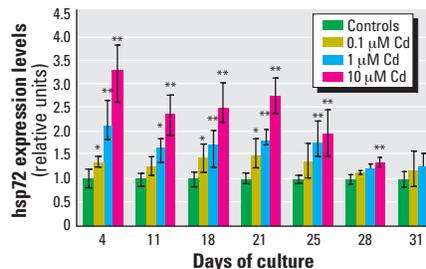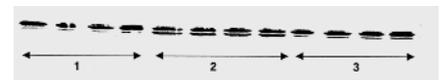

Figure 5. Evolution pattern of the level of hsp72 expression during chronic exposure (31 days) to different Cd concentrations. Values correspond to the mean of three separate experiments, each comprising three cultures for each Cd concentration.
*$p < 0.05$. **$p < 0.01$.

Figure 6. Comparison of the expression of hsp72 (inducible) and hsp73 (constitutive) in (*1*) control A549 cells or grown in presence of (*2*) 10 μM CdCl$_2$ for 3 days or for (*3*) 31 days.
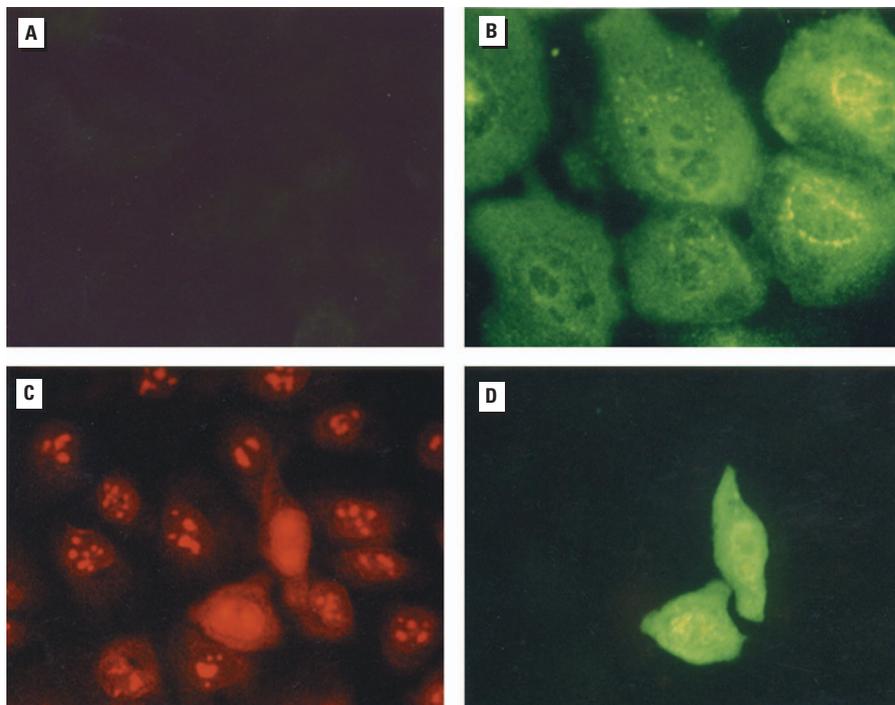
Figure 7. Indirect immunofluorescence analysis of hsp72 in A549 cells of (*A*) control cultures or (*B*) A549 cells exposed for (*C*) 1 hr to 200 μM CdCl₂, or (*D*) for 24 days to 10 μM CdCl₂. Double staining with (*C*) propidium iodide shows (*D*) the nonimmunoreactive cells of the culture.

Figure 8. Indirect immunofluorescence analysis of the number of hsp72-positive cells during chronic exposure (31 days) to different Cd concentrations. Nonimmunoreactive cells were visualized by propidium iodide staining.

Cd induces metallothionein overexpression. Because there is a general agreement that MTs can protect an organism against heavy metal toxicity, we investigated the expression of MTs. Figure 9 (lane C) and Figure 10 (lanes 1–3) show that MT expression was low in control A549 cells. The MT level increased as a function of Cd concentrations ranging from 0.1 to 5 μM (Figure 9A) and 10 μM [(Figure 10); lanes 4–6]. Surprisingly, no further accumulation of MT was found after 15-day exposure (data not shown) or 21-day exposure to Cd (Figure 9B). Similar results were found with 10 μM Cd (Figure 10), and no major difference in the accumulation of MTs was found between A549 cells exposed for either 3 days (lanes 4–6) or 31 days (CD31) (lanes 7–9).

When CD31 cells were replaced in standard culture medium (without Cd) for 15 days [(Figure 10); lanes 10–12], MT expression decreased to levels similar to those found in controls (lanes 1–3). These levels show that MT overexpression did not account for the observed Cd resistance of CD31 A549 cells.

Discussion

Chronic exposure to low levels of toxic metals is an increasing global problem. Cadmium has become ubiquitous in the biosphere largely because of its use in industry and its presence in cigarette smoke. Epidemiologic evidence suggests that Cd exposure may cause pulmonary damages such as emphysema and lung cancer (*26*). In mammals, soluble Cd salts can accumulate and result in cytotoxicity to most organs including liver, kidneys, lungs, bones, and reproductive organs (*27*). The effects of Cd on the stress response genes and the induction of hsp have been reported (*11,14–28*). This induction corresponds to a defense mechanism whereby cells detect damaged cellular components and activate appropriate repair pathways. Increased expression of the hsp70 stress proteins appears to be a potential early marker of toxic exposure (*14,27–29*).

We performed this study to determine whether low Cd concentrations that induced a decrease of A549 cell growth rate, but not cell mortality, triggered an hsp72 overexpression which could be used as an index of environmental pollution by Cd.

We were able to determine the lowest doses of Cd that led to an increase of hsp72 expression in A549 cells for given durations of exposure. For short-term exposures (2–6 hr), an overexpression of hsp72 was observed for Cd concentrations higher than 50 μM. This result correlates with Fischbach et al. (*28*), who reported an overexpression of a transfected human growth hormone gene sequence driven by the hsp70 promoter in NIH/3T3 cells after a 5-hr exposure to 50 μM Cd.

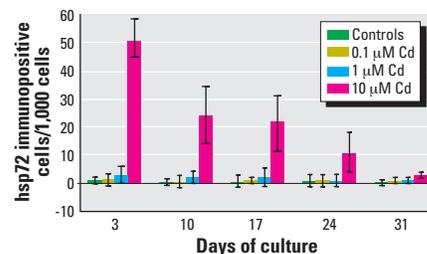Our results support the idea that hsp72 might be considered an early biomarker of short-term exposure to Cd. Overexpression can be observed as early as 6 hr after acute exposure (1 hr, 50 μM); it was maximal 20 hr later. However, in this case, such a dose can only result from an emergency situation. For exposures lasting longer than 48 hr, Cd concentrations as low as 0.1 and 1 μM induced a significant increase of the hsp72 level in A549 cells. We obtained similar results using a human-derived hepatoma cell line (HepG2) or normal human fibroblasts. Timblin et al. (*14*) report a 2.5-time increase of expression of hsp72/73 and hsp78 in lung epithelial cells exposed for 48 hr to 10 μM CdCl₂, which suggests that longer-lasting exposure to lower doses could also stimulate the expression of these genes in epithelial cells.

The concentration of 0.1 μM Cd (11 μg/L) is close to the maximal admissible values in blood as established by regulations dealing with occupationally exposed workers (5–10 μg/L). A blood Cd value of 10.8 μg/L was detected in some workers after years of occupational exposure (*30*). It would be interesting to know whether mononuclear blood cells of exposed workers expressed a level of hsp72 higher than nonexposed individuals.

Cd emitted into the atmosphere contaminates the environment and enters the human food chain. A Swedish study reported that the edible parts of an average-sized crab contain 850 μg Cd (*31*). Thus, the high crab consumption rates reported for Norway (i.e., an average consumption of three crabs/week) (*32*) could result in a weekly Cd intake of 2,550 μg. Although the gastrointestinal fractional absorption of Cd is low (usually < 10%) (*33*), such intake can be important when considering the long biologic half-life of the metal (30 years) (*34*) and the fact that it accumulates in various tissues as intestinal mucosa, liver, and kidney (*35*). These organs account for more than 70% of the total body burden Cd in mice after long-term Cd exposure, and excretion of absorbed Cd is extremely low (*36*). These results show that Cd concentrations in the blood are probably lower than that inside some organs.

Another group of people exposed to relatively high levels of Cd are smokers. In

Table 1. Effects of Cd on A549, CD31, and CD 31–15 cell growth.

	Cell growth inhibition (%)/cells grown in Cd-free medium					
	A549 cells		CD31		CD31–15	
Experiment	10 µM	25 µM	10 µM	25 µM	10 µM	25 µM
1	53 ± 6*	77 ± 5*	3 ± 3	11 ± 6	6 ± 3	12 ± 4
2	44 ± 7*	62 ± 4*	1 ± 6	5 ± 2	3 ± 3	8 ± 3
3	53 ± 3*	73 ± 6*	4 ± 3	15 ± 3	5 ± 3	16 ± 4
4	55 ± 5*	71 ± 7*	10 ± 4	17 ± 4	13 ± 4	20 ± 5*

Percent of cell growth inhibition in four experiments. Values are means of three cultures ± SE. *$p < 0.01$.
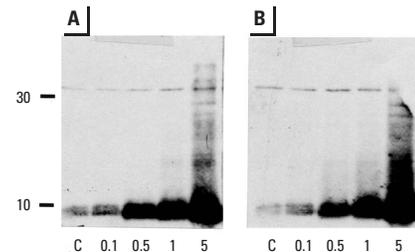

Figure 9. MT immunoblots of A549 cells grown for (A) 4 or (B) 31 days in the presence of Cd concentrations ranging from 0.1 to 5 µM. C, controls. SDS-PAGE was performed using 18% acrylamide gel. Immunoblotting was performed according to the procedure of Mizzen et al. (25); it includes 2 mM CaCl$_2$ in electrophoretic transfer buffer and glutaraldehyde fixation of nitrocellulose membrane.

Figure 10. Expression of the MTs in A549 cells controls (lanes 1–3) or grown for 4 days (lanes 4–6) and 31 days (lanes 7–9) in the presence of 10 µM Cd. Lanes 10–12 correspond to A549 cultivated for 1 month in the presence of 10 µM Cd and put back in Cd-free medium for 15 days.

addition to Cd, tobacco smoke contains many other volatile compounds (benzene, toluene, aldehydes, etc.) that could potentially induce overexpression of hsp. We did not find any change in hsp72 expression related to some of these constituents.

It could be useful to investigate any hsp72 overexpression in human cells with the goal of predicting chronic environmental exposure to low doses of Cd. Surprisingly, our study shows that the hsp72 overexpression induced by short-term exposure to Cd levels ranging from 0.1 to 10 µM gradually decreased through long-term exposure. After 1 month of exposure, A549 cells seemed to have adapted; they showed a higher resistance to Cd. A Cd concentration 5 times higher was necessary to obtain the same growth inhibition as in the first 4-day exposure. Moreover, in cell populations chronically exposed to 10 µM Cd, a small number of cells (5%) that had high hsp72 levels at the beginning of the experiment disappeared later. Because several authors showed that Cd was apoptotic in the human T-cell line (37), testicular tissue (38), and kidney cells (39), it is possible that this high reactivity appears in cells entering apoptosis and, thus, that we selected the most resistant cells in our cultures.

Cd-resistant cells have been isolated from various mammalian cell lines (13–15) or from Cd-adapted animals (40). MT overexpression seems crucial in mediating the Cd-resistant phenotype. MTs, first identified as Cd-binding proteins (41), are induced in
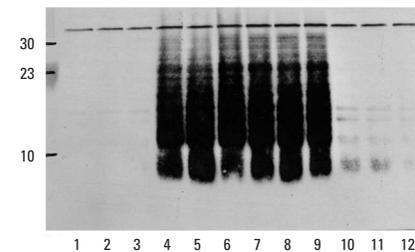
the presence of metals, and it has been proposed that they protect the organism from heavy metal toxicity (18). Therefore, the expression of MTs was investigated in our control and Cd-exposed A549 cells. As compared to the low MT basal level of normal A549 cells, a dose-related increase of MTs was found after a 4-day exposure to Cd concentrations ranging from 0.1 to 10 µM. Surprisingly, no further accumulation of MTs was found in cells grown for 1 month in the presence of Cd. Therefore, no time-dependent increase of intracellular MT levels was found in relation to the decrease of both Cd toxicity and hsp72 induction during 1 month of chronic exposure. Accordingly, we found that Cd-resistant cells replaced for 15 days in a Cd-free medium displayed MT levels which almost decreased to control values. However, they maintained their resistance to subsequent Cd exposures.

Hart et al. (40) found that alveolar macrophages isolated from rats, when repeatedly exposed to Cd aerosols, showed increased resistance to reactive oxygen species associated with an elevation in glutathione peroxidase and glutathione reductase enzyme activities. We found that glutathione contents were similar in both control and Cd-resistant cells (data not shown).

Conclusions

Our work supports the idea that hsp72 might be considered a biomarker of severe acute (50 µM) or occupational (0.1 µM) exposure to Cd. However, in populations living in contaminated sites, it is possible that a certain cell adaptation occurs, with a

decrease in hsp72 induction as a consequence. MT level increased in a similar dose-related manner, and MT overexpression was constant throughout Cd chronic exposure; therefore, MT overexpression appears to be a more reliable indicator of Cd pollution than hsp72 induction.

Moreover, our work suggests that the cytotoxic threshold concentration of Cd is < 0.1 µM (11 µg/L), and that the maximum biologic exposure limit of 5 µg/L recommended by the World Health Organization (42) should be reconsidered.

We agree with Ryan and Hightower (43), who think that problems related to chemical exposures can be identified earlier and with greater sensitivity by using biomarkers at the molecular level, rather than by measuring biomarkers linked with changes at higher levels of organization (renal dysfunctions, reproductive failure, etc). Indeed, as stated by Goering et al. (27), stress protein synthesis induced in rat liver by Cd precedes hepatotoxicity.

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