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LOW CONCENTRATIONS OF MERCURY INDUCE CHANGES IN ION COMPOSITION OF CULTURED MYOBLASTS

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Abstract

The effects of low concentrations (1 pM, 1 nM, 1 μM) of mercuric chloride on ion distribution in cultured myoblasts were analysed by energy dispersive X-ray microanalysis. An increase in intracellular sodium concentration was observed five minutes after addition of $HgCl_2$ to the culture medium. This increase was dose dependent and accompanied by a transient decrease in potassium concentration. Exposure to 1 nM and 1 μM $HgCl_2$ led to a two-fold increase in the cytoplasmic chlorine concentration. The higher $HgCl_2$ concentration (1 μM) induced morphological alterations in the form of cell membrane blebs, perforations and shrinkage or flattening of the myoblasts. It was concluded that even low concentrations of mercuric chloride cause elemental and morphological changes in cultured myoblasts, which may reflect effects of the metal on membrane permeability.

Key Words: Mercury, myoblasts, cell culture, ions, X-ray microanalysis.

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Introduction

Exposure to mercury occurs via many different routes and in different forms. The major source of inorganic mercury exposure is from amalgam fillings and the most important source of organic mercury is from fish. During mastication, consumption of hot drinks and restoration procedures, Hg-vapor is released from the fillings (Langworth *et al.*, 1988; Björkman and Lind, 1992).

Despite its toxicity, mercury has a low carcinogenic potential. The largest pools of mercury in the body are in the kidney and liver but metal accumulation occurs also in mucous membranes, skin and spleen (for review see Nordlind, 1990) and occipital cortex (Nylander *et al.*, 1989). In spite of the high frequency of amalgam fillings among most populations in the western world, there is no evidence that mercury vapor released from the fillings has systemic toxicological effects or causes systemic diseases (WHO, 1991; Public Health Service, 1993).

The clinical manifestations of mercury poisoning include the dysfunction of the central nervous system, muscle weakness, renal failure and osteomalacia. The underlying mechanisms of action of the toxicological properties of mercury are not fully elucidated. Apart from the numerous cytotoxic effects (Christie and Costa, 1984; Clarkson *et al.*, 1988), mercury and cadmium have been shown to stimulate cell proliferation (Nordlind and Henze, 1984; Lu *et al.*, 1990; von Zglinicki *et al.*, 1992). Previously, we observed that low concentrations of $HgCl_2$ (< 100 pM) induce DNA-synthesis and activation of proto-oncogene and metallothionein transcription in cultured myoblasts (Wroblewski *et al.*, unpublished observations). Others have demonstrated that $HgCl_2$ can induce skeletal muscle contraction by causing release of Ca^{2+} from the internal pool (Fu *et al.*, 1988).

Energy dispersive X-ray microanalysis (XRMA) has proven to be a useful method for the detection of elemental concentrations at cellular and subcellular level. We have previously described anhydrous preparation and analysis methods that allow detection of ionic changes in

Figures 1-3. Effects of $HgCl_2$ on sodium (**Fig. 1**), potassium (**Fig. 2**) and chloride (**Fig. 3**) concentrations in cultured myoblasts, analyzed by energy dispersive X-ray microanalysis. Time-dependent changes in concentrations (of sodium, potassium and chloride, respectively) after incubations with (**1a, 2a** and **3a**) 1 pM $HgCl_2$; (**1b, 2b** and **3b**) 1 nM $HgCl_2$; and (**1c, 2c** and **3c**) 1 μM $HgCl_2$. Absolute concentrations are expressed in mmol/kg dry weight. Mean and standard deviations. Statistical analysis was carried out using Student´s t-test (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

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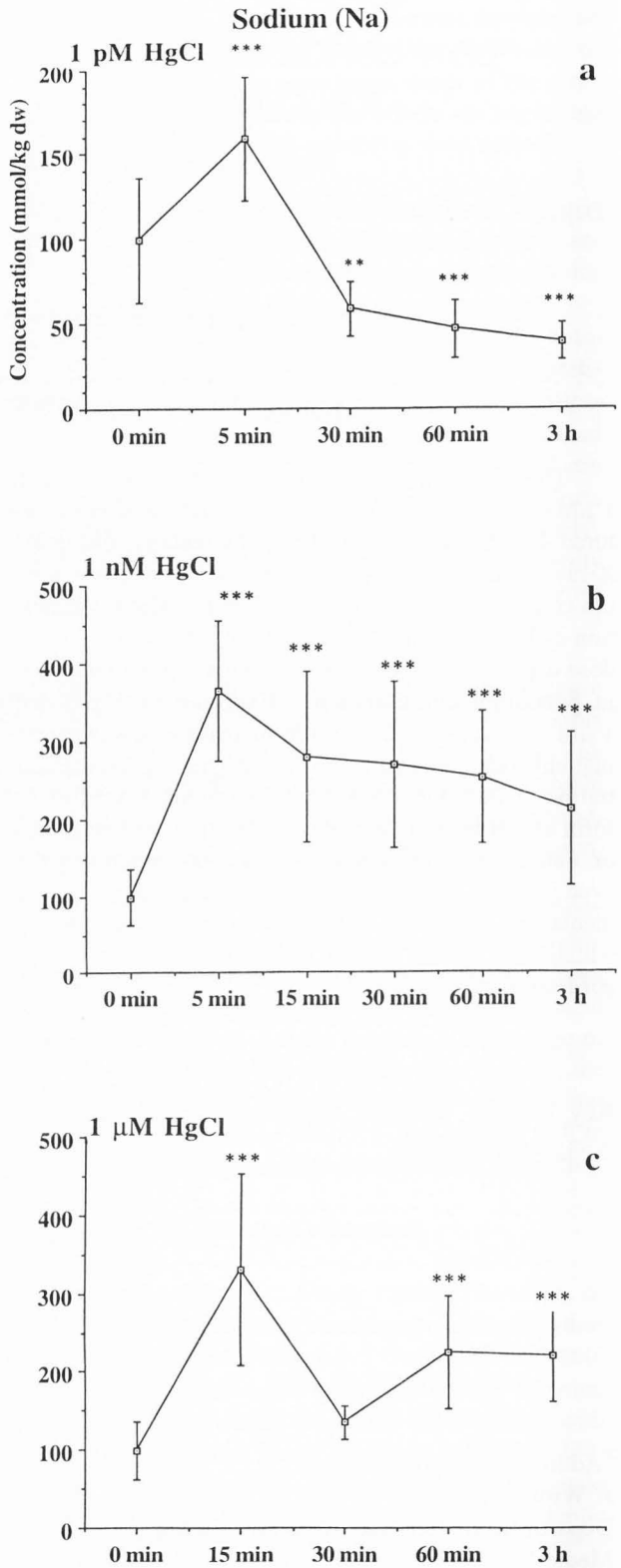
cultured cells by means of XRMA (Wroblewski and Wroblewski 1993, Wroblewski *et al.*, 1983). The aim of the present study was twofold: to investigate the effects of low concentrations of $HgCl_2$ on ionic transport and membrane permeability in L6J1 myoblasts, and secondly to determine whether or not there is a correlation between the metal-induced changes in elemental composition of the cells and membrane morphology.

Materials and Methods

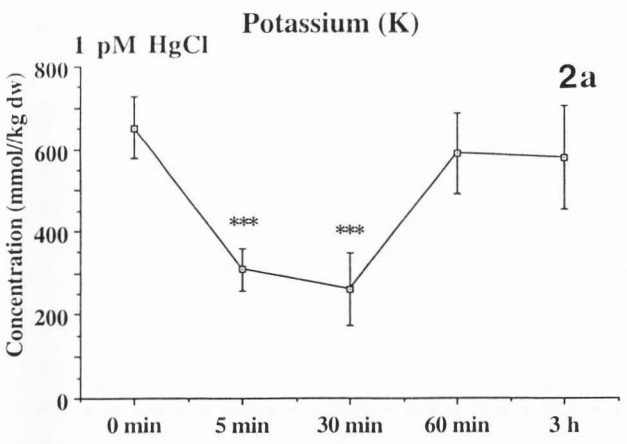
Cell culture

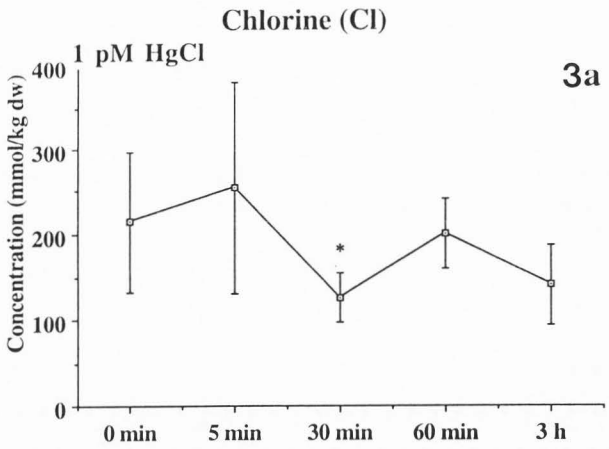
The rat myoblast cell line L6J1 was used (Ringertz *et al.*, 1978). The cells were grown in 9 cm petri dishes (Costar, Cambridge, MA, USA) in Dulbecco´s modified Eagle´s medium (DMEM) containing 5% v/v fetal calf serum (FCS), 20 mM glutamine and 0.1% gentamicin in an atmosphere of 5% CO_2. The subconfluent cultures were detached by 0.25% trypsin, washed with serum-free culture medium by centrifugation, resuspended in serum-containing medium and seeded on titanium electron microscope grids. The grids were coated with a Formvar-film, and sterilized by ultraviolet irradiation prior to seeding, and placed on the bottom of dry petri dishes. Droplets of cell suspension were deposited on the grids according to Wroblewski and Wroblewski (1993). After 2 hours, when the cells had attached, the grids were covered with additional growth medium. On the following day (after 16 hours), the culture medium was changed to fresh medium supplemented with 0, 1 pM, 1 nM and 1 μM $HgCl_2$ (Sigma). The 100 mM $HgCl_2$ stock solution (calculated on the basis of the salt) was prepared in deionized, double distilled water. To obtain the desired $HgCl_2$ concentrations the appropriate amounts of the stock solution were diluted in culture medium. The cells were incubated for 0 minutes to 3 hours $\pm$ $HgCl_2$. At the various time points, the grids were rinsed in distilled water and, after removal of excess fluid by blotting, were cryofixed in liquid nitrogen (LN_2) (Wroblewski and Wroblewski, 1993). The specimens were freeze-dried in vacuum and were carbon coated prior to analysis in the electron microscope.

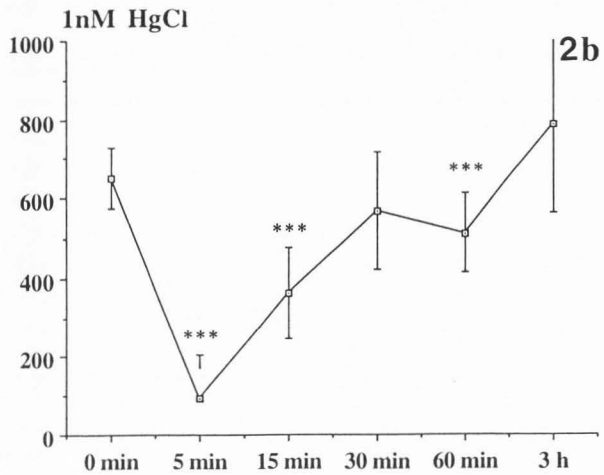


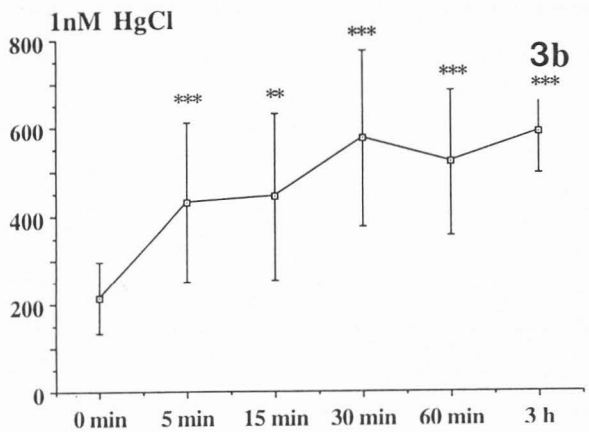
X-ray microanalysis

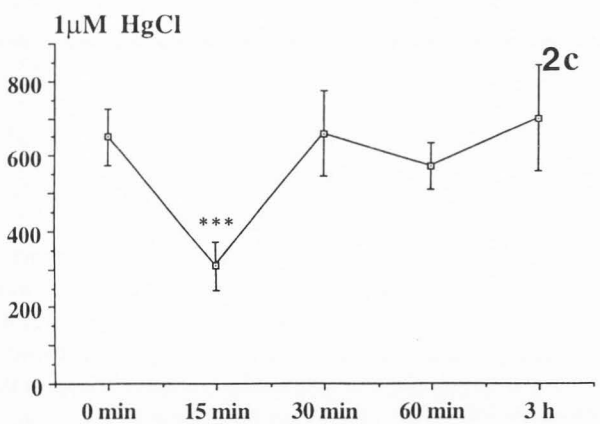
The cells were analysed in a JEOL 1200EX TEM-SCAN electron microscope operated at an accelerating voltage of 100 kV in the scanning transmission mode.

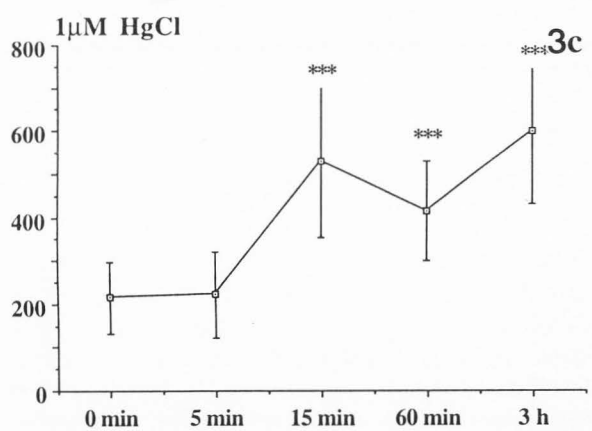












For elemental analysis, a Tracor 5500 energy dispersive XRMA system was used. Analysis was carried out for 60 seconds live time and was restricted to the cytoplasm. Quantification of the absolute concentrations of sodium, potassium and chloride was done according to the method described by Wroblewski *et al.* (1983). Cells from two grids from two separate petri dishes (16-20 cells per grid) from each experimental group were analysed. Sta-

tistical analysis was carried out using paired Student´s t-test to compare treated cells with controls.

Scanning electron microscopy (SEM)

For studies of cell surface morphology, the cells were grown on cover slips (Thermanox) in 24-well plates in serum-containing DMEM. Upon reaching confluency, the cells were incubated for 3 hours with varying concentrations of $HgCl_2$ in medium supplemented with 5% FCS. After completing the incubations, the cells were quickly rinsed in phosphate buffered saline (PBS) solution, pH = 7.4, and were fixed in 3% glutaraldehyde in PBS (pH = 7.4). Subsequently the preparations were washed with buffer (PBS), distilled water, and then dehydrated in graded ethanol, and in acetone. Finally, the specimens were dehydrated in tetramethylsilan and air dried. After coating with 8 nm thick gold layer, the cells were imaged and photographed in a JEOL JSM 820 scanning electron microscope (SEM) operated at an accelerating voltage of 5-10 kV.

Results

The results, summarized in Figures 1 to 3, show that $HgCl_2$, in concentration as low as 1 pM, induces changes in the elemental composition of the cells. Already, five minutes after addition of the $HgCl_2$, a significant, dose-dependent increase in sodium concentration was detected (Fig. 1). In the lowest $HgCl_2$ concentration, the increase was transient, while in 1 nM and 1 μM, it remained at twice the control level for at least 3 hours. The increase in sodium was accompanied by a significant decrease in potassium (Fig. 2). However, potassium levels returned to normal after 30-60 minutes. The changes in sodium and potassium were followed by a three fold increase in chlorine concentration when 1 nM-1 μM $HgCl_2$ was used (Figures 3b and 3c).

Effects of 3 hour incubation with $HgCl_2$ on cell morphology were observed by SEM (Fig. 4). No changes in the cell surface topography could be detected in cells cultured in 1 pM and 1 nM $HgCl_2$ compared with control cells (Figs. 4a, 4b and 4e). At these concentrations, the cells were well spread, large and covered by uniformly distributed short microvilli. Significant changes in the cell morphology were detected first at 1 μM $HgCl_2$ (Fig. 4c); the cell membranes were no longer covered with uniform microvilli, and frequent blebs could be found on the majority of the cells. Furthermore, the homogeneous cell population became heterogeneous with respect to cell size, occurrence of microvilli and blebs, and attachment to the growth support; a subpopulation of cells started to round up and detach from the cover slips (not shown). At 1 mM $HgCl_2$, evidence of massive cell death was observed because only a few cells remained on the cover slips; the remaining cells were rather large, flat and had porous, perforated membranes with no microvilli (Fig. 4d).

Discussion

Mercury is a well known highly toxic substance. At high exposures, the severity of response is usually correlated to the intensity and duration of exposure.

High concentrations of mercury are known to be cytotoxic (Nicholson *et al.*, 1983, Christie and Costa, 1984; Clarkson *et al.*, 1988). Low concentrations, pico- and micromolar, on the other hand, can stimulate DNA-synthesis and proliferation of cells (Lu *et al.*, 1990; Nordlind and Henze, 1984; Wroblewski *et al.*, unpublished observation). Furthermore, picomolar concentrations of $HgCl_2$ induce expression of two proto-oncogenes, c-jun and c-myc (Wroblewski *et al.*, unpublished observation). The molecular mechanisms regulating the effects of low $HgCl_2$ concentrations on cell cycle control have yet not been identified. We observed that mercury induced the influx of sodium and outflow of potassium from the cells. Sodium and potassium concentrations in the cells are regulated by a Na^+/K^+-pump, and malfunction of this pump may lead to the elemental changes observed in the present study. Depolarization of the cell membrane, due to mercury exposure, has been observed in mouse diaphragm (Liu and Lin-Shiau, 1992). It has been suggested that heavy metals such as cadmium, lead and mercury, can mimic the effects of calcium ions on calmodulin expression which subsequently can regulate several kinases and thus stimulate cell division (Lu *et al.*, 1990). It has also been shown that low concentrations of mercury enhance cell membrane conductance for potassium causing transient hyperpolarization, while higher concentrations lead to depolarization of the cell membrane. Thus, mercuric ions possibly activate potassium channels directly (Jungwirth *et al.*, 1991), which, in the present study, could explain the rapid loss of potassium ions from the cytoplasm. Changes in sodium and potassium concentration were associated with an increase in chloride levels. This can be explained by the fact that Hg^{2+} mimics the Cl^- channel blocker 9-anthracene carboxylic acid (Liu and Lin-Shiau, 1992).

The more drastic effects of higher mercury concentrations on the elemental content of the myoblasts were reflected in changes of membrane morphology (Fig. 4). The disappearance of microvilli at $HgCl_2$ concentrations > 1 μM could indicate that changes are occurring in the membrane conductivity of the myoblasts.

In conclusion, both "mitogenic" (1 pM and 1 nM) and "toxic" concentrations (1 μM) of $HgCl_2$ caused transient increase of sodium and loss of potassium from the cells. These changes were dose- and time-dependent and led to alterations in the morphology of cell membranes.

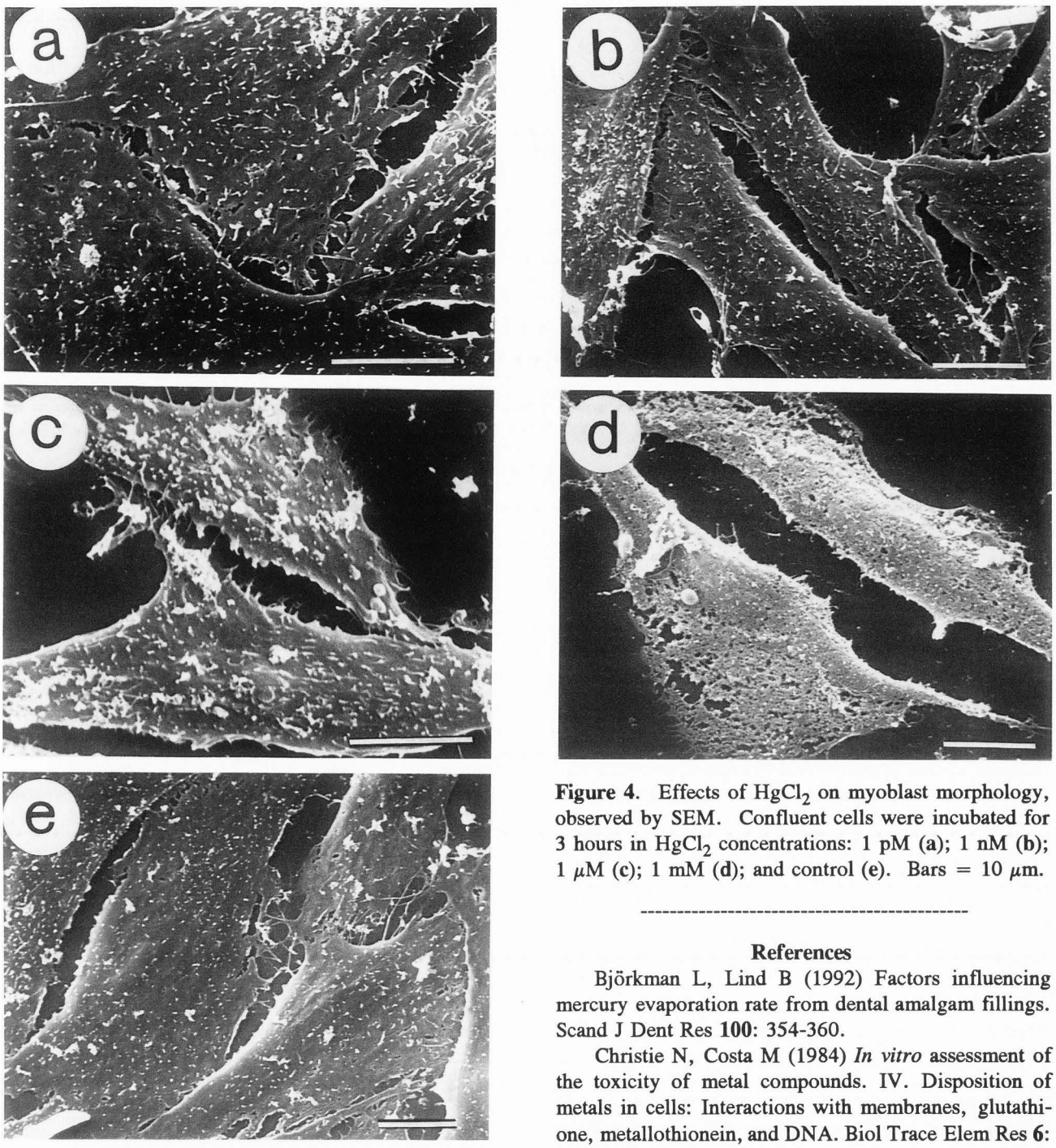


Figure 4. Effects of $HgCl_2$ on myoblast morphology, observed by SEM. Confluent cells were incubated for 3 hours in $HgCl_2$ concentrations: 1 pM (**a**); 1 nM (**b**); 1 μM (**c**); 1 mM (**d**); and control (**e**). Bars = 10 μm.

Acknowledgements

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Discussion with Reviewers

U. Lindh: Mercury is not believed to be carcinogenic, possibly because the DNA damages induced are not repaired to the same extent as, for example, radiation induced damages. Do you think that expression of proto-oncogenes by low-dose exposure to mercury may have implications for the carcinogenicity?

Authors: The fact that $HgCl_2$ can induce expression of proto-oncogenes c-jun and c-myc does not necessarily signify that mercury causes cancer. Proto-oncogenes are also activated in response to the mitogenic stimuli. However, mercuric ion can interfere with thymine. It cannot be excluded that the interaction with DNA may interfere with the replication and transcription processes and thus lead to development of tumors.

U. Lindh: Calcium is not directly the objective of your study but you discuss implications with this element. Depolarization of the membrane caused by an increase of mercury concentration may result in inflow of excess Ca^{2+}, which could then increase the concentration of free Ca^{2+} ions in the cytosol. Could such a mechanism be partly responsible for the cell damage?

Authors: Calcium plays an important role in the induction of programmed cell death, apoptosis. Therefore, it is possible that increased Ca^{2+}-levels, following exposure to $HgCl_2$, may produce irreversible cell damage and cell death.

A.J. Morgan: It is possible that the changes induced by mercuric ions are "latent", in the sense that they are expressed as changes in ion gradients only because you washed the cells with distilled H_2O prior to analysis? This could easily be checked by analyzing Hg^{2+}-treated cryosectioned cells with and without washing.

Authors: We agree that only by analysis of cryosectioned cells one could validate the rinsing method. In a previous and in the present study we have compared effects of different rinsing solutions on the elemental content of cultured myoblasts and found that H_2O-rinse gives reproducible results. The procedure is widely and successfully used by other groups (e.g., Claude Lechene, Thomas von Zglinicki). The fact that the elemental changes induced by $HgCl_2$ are time- and concentration-dependent further confirm the reliability of this preparation technique.

G.M. Roomans: Have the presence of K^+ and Cl^- channels been demonstrated in this particular cell line?

Authors: L6 cells can form myotubes in culture and become contractile. Therefore, we assume that these cells, like other skeletal muscle, express both K^+ and Cl^- channels. Zachar and Hurnak (1994) have described the presence of an outward Cl^- channel in L6 myoblasts.

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