The cytotoxicity of mercury chloride to the keratinocytes is associated with metallothionein expression

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Abstract. There are trace amounts of heavy metals in cosmetics. Heavy metals such as mercury (Hg), which is added to skin-whitening cosmetics, may cause acute or chronic damage to human cells. The aim of this study was to investigate the cytotoxicity of mercury chloride (HgCl₂) to human keratinocytes. The keratinocytes were treated with various concentrations of HgCl₂ and the cell survival fractions were found to be 38.08, 17.59, 12.76, 3.29 and 0.77% when the cells were treated with 0.25, 0.5, 0.75, 1 and 1.5 µM of HgCl₂, respectively. Moreover, we observed that the greatest damage was to the cell membrane. The metallothionein (MT) protein expression was also investigated. MT expression levels increased with increasing concentrations of HgCl₂. The results indicated that MT protects the keratinocytes against HgCl₂-induced toxicity.

Introduction

There are trace amounts of heavy metals in cosmetics. Heavy metals such as mercury (Hg), which is added to skin-whitening cosmetics, may cause acute or chronic damage to human cells. Hg, a divalent metal with no known biological function, may cause several deleterious effects in adults (1,2), as well as in developing organisms (3,4), which primarily involve the central nervous system (5-7) and the kidneys (1,8,9). Young animals seem to be more sensitive to Hg toxicity than adults, particularly during the first days following birth. Hg is also a widespread environmental and industrial pollutant that induces severe adverse effects in humans as well as the environment (10). Its carcinogenic activity has been well-documented. Hg is also known to alter the intracellular redox homeostasis (11,12), which is recognized as a factor that determines cell fate (13). The outcome of cells exposed to Hg-containing compounds depends on the chemical characteristics of the compound, as well as on its dosage, accounting for the various results reported in the literature, ranging from improved cell survival to apoptosis and necrosis.

Keratinocytes have long been considered the structural backbone of the epidermis; however, there is increasing evidence that they play an active role in the pathogenesis of skin damage by heavy metals (14). Available histopathological (15) and cytotoxicological (16-18) studies describing keratinocyte damage by mercury chloride (HgCl₂) are currently limited. This underlines the importance of investigating the direct cytotoxic effects of the metals on keratinocytes, as well as intracellular damage, for which available data are limited.

Metallothioneins (MTs) are ubiquitous, low-molecular weight proteins, rich in cysteine residues. Their high content of sulfhydryl amino acids (~30%) gives these proteins unique metal-binding properties (19,20). Factors such as exposure to toxic or essential metals (3,21-23), stress (24,25), radiation (26) and other agents (27,28), promote the synthesis of these molecules (29). With respect to their biological functions and due to the metal affinity of their sulfhydryl groups, it is believed that MTs possess antioxidant properties (26,30), are involved in the homeostasis of essential metals such as zinc (Zn) and copper (Cu) (20,29) and act as detoxifying agents from metal ions (20,31,32).

In this study, we investigated the cytotoxicity of HgCl₂ to human keratinocytes, using human keratinocyte-derived HaCaT cells as an experimental model. In addition, we focused on HgCl₂-induced HaCaT cell damage and examined the expression of MTs.

Materials and methods

Materials. Human keratinocyte-derived (HaCaT) cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Dulbecco’s modified Eagle's medium (DMEM), heat-inactivated fetal calf serum (FCS), penicillin-streptomycin solution and trypsin-EDTA solution were purchased from Life Technologies Corporation (Carlsbad, CA, USA). Sterile dimethylsulfoxide (DMSO) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and...
HgCl₂ were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Cell culture.** HaCaT cells were grown in DMEM supplemented with heat-inactivated FCS (10%; v/v), streptomycin (100 U/ml) and penicillin (0.1 mg/ml), in a humidified atmosphere of 5% CO₂ at 37°C. The culture medium was changed three times a week. The cells were subcultured following trypsinization and seeded in 6-well plate at a density of 1x10⁵ cells per cm².

**Cells treated with HgCl₂.** The keratinocytes were treated with HgCl₂ (0.25-1.5 µM) at 37°C for 24 h. When the non-treated control cells were grown confluent, the cell groups were prepared for cell viability assay or MT western blot analysis.

**MTT assay.** The cell viability was monitored following treatment with various concentrations of HgCl₂. MTT was used to quantify the metabolically active living cells. Mitochondrial dehydrogenases metabolize MTT to a purple formazan dye, which was measured photometrically at 570 nm using a spectrophotometer (33).

**Western blot analysis for MT protein expression.** Cell homogenates were prepared by sonication of cells in 600 µl of ice-cold lysis buffer, containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 µg/ml PMSF, 1 µg/ml aprotinin and 1% NP-40. Homogenates were clarified by centrifugation at 20,000 x g for 45 min at 4°C. Total protein concentration was determined using the BCA (Bio-Rad, Hercules, CA, USA) assay. Samples (50 µg of total protein) from HaCaT cells treated for 24 h with various concentrations of HgCl₂ were analyzed for human MT proteins, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) in 10-20% gradient gels. Proteins were electrophoretically transferred to nitrocellulose membranes. The resulting membranes were incubated in 2.5% glutaraldehyde for 1 h and then washed 3 times for 5 min in phosphate buffer (8.1 mM Na₂HPO₄, 1.2 mM KH₂PO₄, 2.7 mM KCl, pH 7.4). Monoethanolamine (50 mM) was added to the third wash solution to quench residual glutaraldehyde reactivity. MT proteins were detected by Immun-Star Chemiluminescent Protein Detection Systems (Bio-Rad). A monoclonal antibody to polymerized equine renal MT (Dako, Carpinteria, CA, USA) was used for immunodetection.

**Statistical analysis.** Means ± standard error (SE) were calculated in triplicate. A statistical significance between the groups was determined by the Student's t-test. P<0.05 was considered to indicate a statistically significant difference between the two groups.

**Results**

**Cell survival fractions of HaCaT cells treated with HgCl₂ at various concentrations.** Comparison of cell survival fractions in HaCaT cells treated with HgCl₂ at various concentrations from 0.25 to 1.5 µM is shown in Fig. 1. The cell survival fraction was 38.08% when the keratinocytes were treated with 0.25 µM of HgCl₂. The cell survival fractions were 17.59, 12.76, 3.29 and 0.77%, when the keratinocytes were treated with 0.5, 0.75, 1 and 1.5 µM of HgCl₂, respectively. For each concentration investigated, a linear characteristic concentration-response curve was observed, with decreased cell survival at increasing concentrations of HgCl₂ on a semi-log scale.

**Effect of HgCl₂ on HaCaT cell morphology.** Keratinocytes were treated with HgCl₂ for 24 h or left untreated. The HaCaT
cell morphology is shown in Fig. 2. The cell membrane of untreated cells is clear and intact (Fig. 2A), whereas that of HgCl₂-treated cells is unclear and interrupted (Fig. 2B).

Effect of HgCl₂ on MT expression. MT expression levels in HaCaT cells treated with various concentrations of HgCl₂ are presented in Fig. 3. MT expression levels increased significantly with the increase in the concentrations of HgCl₂.

Discussion

The purpose of this study was to assess the cytotoxicity of HgCl₂ to the keratinocytes, as well as MT expression in HgCl₂-treated keratinocytes. The results demonstrated that exposure of HaCaT cells to HgCl₂ resulted in dose-dependent cell death and distinct cell membrane damage. Reports of Hg poisoning due to exposure to skin-whitening creams, ointments and soaps have increased significantly over the past few years. Furthermore, since people with lighter skin tone may represent a higher status in certain cultures, skin -whitening cosmetics and soaps are widely used by women to enhance their appeal (34-36). Otto et al (36) detected high Hg concentrations in the blood and urine of Balkan refugees of varying ages who had been exposed to a Hg-based skin-whitening ointment.

We have demonstrated that exposure of keratinocytes to HgCl₂ resulted in cell membrane damage. Picoli et al (37) also investigated the effect of HgCl₂ on gap junction intercellular communication (GJIC) in cultured human keratinocytes. They demonstrated that subcytotoxic concentrations of HgCl₂, as low as 10 nM, may cause inhibition of the GJIC. In addition, they demonstrated that HgCl₂-treated keratinocytes exhibited a decrease in free thiols and accumulation of mitochondria-derived reactive oxygen species, albeit no effect on the respiratory chain activity was observed.

This study has demonstrated that MT expression may be induced by HgCl₂ in HaCaT cells. Kramer et al (38) demonstrated that MT may be induced by Hg²⁺ in neuronal cells and induced MT decreases the rate of metal binding to other structures, providing protection against metal toxicity (39). Apart from Hg, MT also plays a role in the homeostasis of essential metals such as Zn and Cu, the detoxification of toxic metals such as Cadmium (Cd) and protection against oxidative stress (40-42). Richards et al (43) and McCormick et al (44) demonstrated that plasma zinc concentrations were related to MT expression, further suggesting an association with cellular zinc homeostasis. Ogra et al (41) demonstrated that cell viability was significantly decreased in MT-null cells compared to wild-type cells by Cu(I)-specific chelator treatment (41). They also showed that MT expression levels were increased by Cu(I)-specific chelator treatment in wild-type cells. Thus, MT was induced under Cu-deficient conditions, in order to maintain the activities of intracellular cuproenzymes, such as cytochrome c oxidase and Cu/Zn superoxide dismutase. Urani et al (42) showed that MT expression was upregulated following exposure to CdCl₂, with a saturation curve at 48 as well as 72 h. High levels of MT possibly confer an acquired tolerance to stress and protection against cell injury, as demonstrated by the low cytotoxicity values.

In conclusion, our results demonstrated that exposure of HaCaT cells to HgCl₂ resulted in significant dose-dependent cell death and cell membrane damage. Moreover, MT expression may be induced by HgCl₂ in HaCaT cells. This suggests that MT protects the keratinocytes against HgCl₂-induced toxicity.

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References


