Atomic force microscopy study on human keratinocytes treated with HgCl₂

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Abstract. Morphological modifications of the cellular membrane of human keratinocytes treated with HgCl₂ at different concentrations were investigated employing atomic force microscopy and Raman microspectroscopy techniques. Important changes in the surface structure of the keratinocytes membrane occur when this chemical treatment is performed. Mercury action gives rise to roughness and hole-like depressions, especially at cytotoxic concentration. Such surface features are mainly localized in peripheral zones of cells. Although the viability assay reveals that the exposure of keratinocytes to HgCl₂ at a concentration of 10⁻⁶ M has no cytotoxic effect, morphological modifications are also evident in cellular membrane at such concentration. Raman microspectroscopy measurements suggest that such morphological modifications are related to modifications occurring in the lipidic layer. Our findings show that atomic force microscopy can be a valid and useful tool in studying the changes in morphology of cells when exposed to chemical stress.

1. Introduction
Mercury, one of the most widely diffused environmental contaminants, has an important role as cytotoxic agents, because of its high affinity to protein-containing thiolic groups [1]. Therefore, mercury exposure causes alterations in protein structure and, consequently, inhibits cellular transport mechanisms [2, 3]. It also disables enzymatic processes by the replacement of essential minerals, such as magnesium, zinc, lithium [4]. Biochemical damages, as inhibition of DNA and RNA synthesis, are also reported because of mercury action [5]. In addition, when mercury or other metals with known toxicity (cadmium, lead) bind to macromolecules, there is often a perturbation of normal biological function. Metal-catalyzed formation of oxygen-derived free radicals can promote pathological disorders, as mutagenicity, carcinogenicity, and aging [6]. Increase in the levels of bioavailable toxic mercury in the environment is, therefore, of great concern to human health.

Environmental sources of mercury include thermometers, barometers, fluorescent lamps, insecticidal products, dentistry amalgams. Mercury exists in different physical and chemical states. In particular, inorganic mercuric chloride HgCl₂ can cause severe irritations to skin and respiratory chain and it can affect the kidneys and central nervous system. Poisoning can result from mercury vapour inhalation, ingestion or absorption through the skin. Mercury is transmitted rapidly throughout the skin and easily crosses cell membranes. So it is interesting the study of cellular damage due to HgCl₂ exposure in epidermal cells, as keratinocytes.

The biological effects due to mercury exposure depend on the exposure dose: at cellular level, the cyto-toxicity is proportional to the HgCl₂ exposure time and concentration. It is desirable to minimize such exposures to levels that do not cause adverse effects. Cytotoxicity is commonly evaluated by biological assays for cell viability. However, such biological assays are not able to evaluate if a non-toxic dose induces structural damages in the treated cells. An estimate of the morphological damages
induced by non-cytotoxic exposure is important because such structural modification can promote pathological diseases.

Investigation of cellular structure has been carried out for several years by means of physical techniques, as atomic force microscopy (AFM) and Raman spectroscopy. AFM is a scanning probe technique producing high resolution three-dimensional images of a wide variety of biological samples, ranging from cells to individual macromolecules. AFM has been recently used to investigate surface modifications in cells exposed to cytotoxic physical agents, as electromagnetic fields [7], and chemical stresses, as ethanol treatment [8]. Raman spectroscopy is a well known analytical technique able to determine the chemical composition and molecular interactions in micrometric samples. There are many applications of Raman spectroscopy to characterize proteins [9] and cells [10].

In this work, the morphological modification in cellular membrane of human keratinocytes exposed to HgCl₂ at different concentrations is investigated. Our results show that morphological modifications are evident in cellular membrane also when HgCl₂ exposure occurs at non-cytotoxic concentrations. Raman spectroscopy measurements at microscopic resolution reveal that such morphological modifications are related to modifications occurring in the lipidic layers.

2. Materials and experimental methods

2.1 Cell cultures. Human keratinocytes HUKE is a normal cell line originated from human skin. The cells were grown in Epilife medium, supplemented with Keratinocyte Medium Supplement, at 37°C, 5% CO₂. Human keratinocytes were treated with increasing doses of HgCl₂ for 24h. Treated and control cells used for every experiment belong to the same flasks and generation.

The Trypan blue method was used to evaluate the doses of HgCl₂ having cytotoxic effect. This method estimates cell viability by means of plasma membrane integrity. Cells with a damaged cell membrane (necrotic cells) stain blue, whereas cells with an intact plasma membrane (healthy or apoptotic cells) remain unstained. Cells are harvested with trypsin and a small aliquot of the cell suspension is diluted with an equal volume of 0.4% Trypan blue solution (Sigma). Cells are then counted under a phase-contrast microscope.

The samples for AFM imaging and Raman microspectroscopy consisted of cells cultured, until confluence, on poly-lysine coated glass coverslips. After the 24h exposition, control and treated cells were washed three times in PBS and then fixed in paraformaldehyde 3.7% in PBS. Before AFM imaging and Raman measurements cells were rinsed two times in PBS.

2.2 AFM measurements. A Perception Atomic Force Microscope (built by the Assing company, Italy) was used for AFM imaging. Mainly, it consists of a stainless-steel unit made of two separable cylindrical supports equipped with a vibrational isolation system. The lower unit contains the sample holder mounted at the top of a piezoelectric scanner having a maximum x-y scan range of 31x31 μm, at a z-range of 8 μm; a micrometric x-y-z motor controlled translator permits fine moving of the sample. The upper unit contains a cantilever holder, the mirror deflection system and a four sector position sensitive photodiode, used as deflection detector. An electronic feedback loop is used to integrate the optical signal and maintain a constant cantilever deflection during the acquisition.

The measurements were performed in air (at room temperature and 50%-60% relative humidity), with the microscope working in the weak repulsive regime of contact mode with force ~1nN from zero cantilever deflection. Gold coated Si₃N₄ cantilevers with a spring constant of 0.05N/m and a statistical apical radius of 5-20 nm were used. Constant force images were acquired with a scan rate of 3.0-4.0 s/row.

Experimental data have been treated by only background subtraction, and reproducibility (including the absence of sample’s damage due to the measurement procedure) has been successfully tested by imaging the same cells many times. To estimate the morphological modifications of HgCl₂ treated cells compared to control ones data were collected from 20 cells for each treated and control sample, of three different experiments.
2.3 Raman measurements. The Raman spectra were recorded at room temperature by means of a Raman confocal microspectrometer apparatus using the 488 nm line of an Ar ion laser as excitation source and a notch filter to suppress the laser scattered light. The laser beam was focused, by an Olympus optical microscope with a x100 oil immersion objective, through the glass coverslip on the fixed cells in PBS solution. A diffraction limited spot of about 1 μm diameter resulted at the focal plane. The laser power on the sample was about 3 mW. The acquisition time was 2 minutes for each cell. The Raman signal was detected by means of a cooled CCD. The mean spectral resolution was 4 cm⁻¹. The Raman spectrum of each type of cell was calculated as the average of the Raman spectra from 10 different cells randomly chosen, on each sample, of three different experiments. The Raman spectra were background corrected by subtraction of signal from PBS solution and glass coverslip.

3. Results and discussion
The effects of HgCl₂ toxicity on the keratinocytes were investigated by measuring their viability with the trypan blue assay after exposing these cells at different HgCl₂ concentration for 24h. The viability level of untreated control cells was very high (94%). When the cells were exposed to HgCl₂ at different concentration from 10⁻⁸ M to 10⁻⁶ M, the viability remained at high level (87%, 93%, 91%, 89% for exposure to 10⁻⁸ M, 10⁻⁷ M, 10⁻⁶ M and 10⁻⁵ M HgCl₂, respectively). Instead, when the keratinocytes were exposed to 10⁻⁴ M HgCl₂ their viability dramatically declined to 2%, indicating that such level of treatment induced cell death. In addition, the optical image (not shown here) shows that the treatment at this high concentration has removed a high percentage of cells from the glass coverslip. Therefore, from the viability test 10⁻⁴ M HgCl₂ was estimated as cytotoxic dose.

As the aim of this work is to reveal morphological modification in cellular membrane of human keratinocytes exposed to HgCl₂ at non-cytotoxic concentrations, we have considered, for AFM and Raman spectroscopy investigation, untreated cells, 10⁻⁴ M and 10⁻⁶ M HgCl₂ treated cells as control, treated at cytotoxic and non-cytotoxic dose sample, respectively.

Figure 1 shows the AFM contact-mode topography of typical unexposed keratinocytes. Such control cells result highly domed and turgid, in the typical polygonal epithelial shape. The surface appears very smooth and lacking of roughness and holes, as can be deduced by the cross-section of topographic profile.

In contrast, the exposure to a cytotoxic dose of HgCl₂ causes an evident damage to the plasmatic membrane, as shown in the topography of a typical 10⁻⁴ M HgCl₂ exposed cell in Figure 2. All these heavily treated cells appear emptied, except a small area, corresponding to nuclear region [11]. They present hole-like depressions which are distributed on the whole membrane surface. The cross sections show that holes and surface roughness are deeper in peripheral zones than in central ones.

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**Figure 1.** AFM image in contact mode topography and cross-section of a typical control keratinocyte cell. The color scale is reported on the left hand side. The scale bar is 5 μm.
Figure 2. AFM image in contact mode topography and relative cross-sections of a typical keratinocyte cell exposed to $10^{-4}$ M HgCl$_2$ for 24h. The color scale is reported on the left hand side. The scale bar is 5 $\mu$m.

Alterations of plasmatic membrane are also evident in keratinocytes treated at not cytotoxic dose, as can be deduced from the topography of a typical $10^{-6}$ M HgCl$_2$ exposed cell in Figure 3. Although such cells result similar to control ones as for the polygonal shape and turgid appearance, the cross-section of topographic profile clearly shows that the cell surface is slightly rough. These alterations are only a partial damage of plasmatic membrane, as confirmed by the results of the viability assay.

Hence the treated keratinocytes, both at $10^{-4}$ M HgCl$_2$ citotoxic concentration and at $10^{-6}$ M HgCl$_2$ not cytotoxic concentration, show plasmatic membrane alterations compared to control ones. Such damage results proportional to the HgCl$_2$ concentration.

Figure 3. AFM image in contact mode topography and cross-section of a typical keratinocyte cell exposed to $10^{-6}$ M HgCl$_2$ for 24h. The color scale is reported on the left hand side. The scale bar is 5 $\mu$m.
Figure 4. Raman spectra of typical untreated keratinocyte cells (control) and HgCl$_2$ treated cells. The labels indicate the attribution of each Raman feature, according to [10,12]. Phe: phenylalanine; Tyr: tyrosine; p: protein; l.: lipids; str: stretching; def.: deformation; twist: twisting; glass: coverslip contribution. The vibrational modes involving lipids are underlined. The vertical arrows below each label indicate the spectral position of the Raman feature. Spectra have been shifted vertically for clarity.

Table 1. Ratio between the intensity of 1449 cm$^{-1}$ Raman peak, due to lipids deformation mode, and the intensity of 1660 cm$^{-1}$ Raman peak, due to amide I vibrational mode.

| HgCl$_2$ dose | (1449/1660) Intensity |
|---------------|-----------------------|
| control       | 0.75                  |
| 10$^{-6}$M    | 0.71                  |
| 10$^{-4}$M    | 0.60                  |

The presence of cellular damage in plasmatic membrane of the keratinocytes after HgCl$_2$ treatment at not cytotoxic dose is confirmed by Raman micro-spectroscopy measurements. A comparison among Raman spectra from control keratinocyte cell and Raman spectra from HgCl$_2$ treated cells is shown in Figure 4. The Raman spectra were collected within the spectral region from 600 to 1800 cm$^{-1}$. This region is known as the molecular fingerprint region and provides the most interesting information about the functional groups inside the cell.

The Raman spectra of keratinocytes show mainly contributions from proteins and lipids cellular components. All the spectra exhibit similar peak structures, with variations between cell types occurring in some peak intensities. In particular, the Raman intensity of the peaks at 1449 cm$^{-1}$ and 1301 cm$^{-1}$, which are both related to CH deformation modes in lipids, decreases with respect to the Raman intensity of proteins peaks when the HgCl$_2$ concentration increases. In fact, the ratio (1449/1660) between the Raman intensity of the 1449 cm$^{-1}$ band, related to lipid CH vibrational mode, and the Raman intensity of the band at 1660 cm$^{-1}$, related to the protein amide I vibrational mode,
decreases with increasing HgCl₂ treatment, as reported in Table 1. This behaviour can be related to structural damage in cellular membrane, due to the breaking of the membrane lipidic layer.

4. Conclusions
The structural damage due to 24h exposure of human keratinocyte cells to HgCl₂ has been studied as a function of the concentration of mercuric chloride. Cell viability was found to remain high up to 10⁻⁵M HgCl₂ concentration but decreased quickly as the concentration rises to 10⁻⁴ M: therefore, this concentration can be considered as toxic one. Nevertheless, AFM results show that morphological alterations of the cellular membrane begin also when exposure at not cytotoxic concentrations occurs. Raman measurements correlate these morphological modifications to structural alteration of the membrane lipidic layer. The interaction of mercury with lipid molecules probably occurs through the increase in cellular concentration of reactive oxygen species (ROS), as hydroxyl radical (HO), superoxide radical (O₂⁻) or hydrogen peroxide (H₂O₂) as a result of mercury action [13]. ROS interaction with lipids causes plasma membrane peroxidation, which consists in a free radical chain reaction by which molecular oxygen is incorporated into constituent polyunsaturated fatty acids to yield lipid hydroperoxides [14]. In particular, this peroxidation process causes a decrease of CH₂ bonds in lipids. The lipid hydroperoxides react with other lipids giving rise to an auto-oxidation cycle that damages deeply cellular membrane [15]. Our work can be considered a preliminary one about the morphological modification characterizing the cellular membrane of keratinocytes when chemical stress occur.

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References
[1] Hughes WL, *Ann. N.Y. Acad. Sci.* 65, 454 (1957).
[2] O’Halloran TV, *Science* 261, 715 (1993).
[3] Goyer RA, *Am. J. Clin. Nutr.* 61 (suppl. 3) 545S (1995).
[4] Danielson BR, *Neurotoxicol. Teratol.* 18, 129 (1984).
[5] Khera KS et al., *Teratology* 8, 293 (1990).
[6] Schurz F, Sabater-Vilar M, Fink-Gremmels J, *Mutagenesis* 15, 525 (2000).
[7] Rieti S, Manni V, Lisi A, Giuliani L, Sacco D, D’Emilia E, Cricenti A, Generosi R, Luce M and Grimaldi S, *Journal of Microscopy* 213, 20 (2004).
[8] Canetta E, Adya AK and Walzer GM, *FEMS Microbiol. Lett.* 255, 308 (2006).
[9] Tuma R, *Journal of Raman Spectroscopy* 36, 307 (2005).
[10] Notingher I, Verrier S, Haque S, Polak JM and Hench LL, *Biopolymers* 72, 230 (2003).
[11] unpublished results
[12] Uzunbajakava N, Lenferink A, Kraan Y, Willekens B, Vrensen G, Greve J and Otto C, *Biopolymers* 72, 1 (2003).
[13] Kim SH and Sharma RP, *Toxicology in Vitro* 17, 385 (2003).
[14] Halliwell B and Gutteridge J: “Free Radicals in Biology and Medicine”, Oxford Press, Oxford (1999).
[15] Alvarez JG and Storey BT, *Biol. Reprod.* 30, 323 (1984).