Electromagnetic Biology and Medicine, 25: 281–289, 2006

ISSN 1536-8378 print
DOI: 10.1080/15368370601044598

informa
healthcare

ELF Non Ionizing Radiation Changes the Distribution of the Inner Chemical Functional Groups in Human Epithelial Cell (HaCaT) Culture

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Human skin cell culture (HaCaT) that has been exposed to an AC magnetic field undergoes detectable changes in its biochemical properties and shapes. Such changes were observed by infrared wavelength-selective scanning near-field optical microscopy with a resolution of 80–100 nm. We specifically investigated the changes in the distribution of the inner chemical functional groups and in the cell morphology induced by a 24 h exposure to a 1 mT (rms), 50 Hz sinusoidal magnetic field in a temperature regulated solenoid. These results further accentuate the crucial questions, raised by several recent studies, about the impact of low-frequency electromagnetic field on human cells.

Keywords ELF; IR; NIR.

Introduction

Infrared (IR) spectroscopy is a potentially powerful tool for identifying the chemistry of biologically active molecules in cells. Several commercially available IR microscopes using standard thermal sources have been developed in recent years to take advantage of such radiation with many applications in surface

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science, solid state physics, and biology (Carr et al., 1998). However, until recently, practical implementation of intracellular IR imaging has been problematic because of the diffraction limit of conventional infrared microscopy, which gives a spatial resolution not better than a few microns.

The advent of scanning near-field optical microscopy (SNOM) (Betzig and Trautman, 1992; Cricenti et al., 1998) has augmented the usefulness of IR spectroscopy at the microscopic level. In fact, by coupling a SNOM with a tunable IR source (usually a laser in this region) it is possible to overcome the diffraction limit in the infrared region (Tuncel et al., 1993).

Tunability of the laser is extremely important since the biochemical cellular compounds have their own vibrational "fingerprints" in the absorption of light, in fact, their frequencies lie in the mid-infrared wavelengths from 3 μm to about 11 μm (spectral range between 3,300 cm^{-1} and 900 cm^{-1}). In particular, the following wavelengths are particularly interesting for determining the inner chemical composition of a cell:

$\lambda = 3.04\,\mu m$ (the asymmetric CH_2 stretching mode (Jamin et al., 1998) corresponding to lipids).

$\lambda = 6.45\,\mu m$ (II band due to the C-N stretching mode and to a C-N-H bending mode, corresponding to proteins (Jamin et al., 1998))

In this way, once the optimal wavelengths are found corresponding to the distribution of functional groups within a cell, it is possible to map the distribution of functional groups (proteins, lipids, and nucleic acids) inside a single cell with a nanometric resolution and to determine possible chemical differences between perturbed and unperturbed cells.

There have been several reports in the scientific literature suggesting a link between exposure to electric and magnetic fields and modification of biochemical parameters on human cells (Adey, 1981, 1993; Ahlbom et al., 2000). One possible target affected by magnetic fields are all the intracelluar biochemical reactions that involve more than one unpaired electron. Most enzymes do not involve radical intermediates and should be unaffected by a change in magnetic field. However, more than 50 enzymes are believed to generate free radical intermediates during catalysis and may be subject to the influence of external magnetic fields. One of the possible mechanisms of how an applied magnetic field, that is weaker in strength than typical hyperfine interactions, can influence the yields and kinetics of recombination reactions of free radicals in solution, the so-called low field effect, is a coherent superposition of degenerate electron-nuclear spin states. A weak applied magnetic field causes these states to oscillate, leading to coherent interconversion of singlet and triplet electronic states of the radical pair and hence changes in the yields of recombination products and of the free radicals that escape into solution. For singlet geminate radical pairs, the low field effect leads to a boost in the concentration of free radicals, which may be relevant in the context of in vivo biological effects of electromagnetic fields.

We have recently reported experiments by fluorescence SNOM and AFM microscopy on HaCaT cells exposed to a 50 Hz magnetic field at a flux density of 1 mT. Data obtained show that the cells undergo a modification in shape and morphology with an increase of the areas of adhesion between them, together with

a corresponding increase of segregation of the sulphur rich protein adhesion marker β4 integrin in the cell membrane (Rieti et al., 2004).

This spontaneously immortalized human keratinocytes cell line, HaCaT, is derived from adult skin, and maintains full epidermal differentiation capacity (Boukamp et al., 1988) after 24 h exposure to a magnetic flux density 1 mT, at a frequency of 50 Hz. Keratinocytes are the principal components of skin, which can be considered an organ that covers our body and protects it from chemico-physical injuries. Particularly, the more external stratus of skin, epidermidis, is mainly constituted by keratinocytes, so that they are the first cell to which the effects of the interaction with the external agents is transferred. For this reason keratinocytes seem to be an excellent model of analysis to study biochemistry interferences eventually caused by the interaction with environmental and/or artificial agents like extremely low frequency (ELF) electric and magnetic fields.

In this article we present a study of HaCaT human keratinocytes cell line, control (non exposed), and 50 Hz–1 mT exposed cells, using a reflection SNOM system coupled with a tunable IR light source, where the entire sample is illuminated by the IR light and the reflected signal is collected.

IR-SNOM images have been collected at different wavelengths to detect specific vibrational modes, characteristics of the chemical constituents of the cell. In this way it is possible to analyze the biochemical variations eventually suffered by cells exposed to a physical agent such as extremely low frequency (ELF) electric and magnetic fields. The biochemical variations were observed with an optical resolution of ~80 nm and were also accompanied by a change in shape.

Methods

Cells

HaCaT is an immortalized cell line derived from human skin (Boukamp et al., 1988). Cells were grown in D-Mem (GIBCO Laboratories, Scotland) supplemented with 10% Foetal Calf Serum (Pardali et al., 2000; Pece and Gutkind, 2000) and antibiotics (110 IU/ml of penicillin and 100 μg/ml of streptomycin) at 37 ± 0.1°C, 5% CO_2, and sub-cultivated twice a week at a 1:5 ratio. Control and exposed cells used for every experiment were from the same flasks and generation.

Exposure Solenoid

Cells were exposed for 24 h to a sinusoidal 50 Hz magnetic field at a flux density of 1 mT (rms) in a temperature regulated solenoid. Temperature regulation 37 ± 0.3°C and 5% CO_2 was provided. Temperature was continuously recorded by a Hanna HI 9274 OC printing thermometer within the center of the solenoid, and was maintained in the 37°C ± 0.3 range. The solenoid was placed in a cell incubator while control cells were run in a second incubator of the same make and in the same conditions. As a double check, in a subsequent experiment the control sample was placed in the cell incubator containing the solenoid with no field, in the same conditions of the exposed one. No differences were detected between control and sham cells. All experiments are made under blind conditions. The solenoid manufacture has been published elsewhere (Santoro et al., 1997). The main body of the solenoid is a cylinder in concrete-asbestos 2 cm thick and has a diameter of 20 cm and a height of 40 cm. It is made of 1,200 turns of 2 mm diameter. Copper

wire wound in three layers in continuous forward/backward fashion. It is driven from the 50 Hz power mains through a variable autotransformer and generates a flux density of 1 mT (rms) for an applied voltage of 12 Volts (rms).The solenoid is then placed into a cell incubator with its center ventilated by the fan for appropriate air circulation. The modest heat due to Joule effect is efficiently dispersed by the continuous forced ventilation in the CO_2 incubator. As stated above, in all the experiments the temperature within the solenoid measured at the level of the sample was never out of the range 37 ± 0.3°C. Since in exposed samples, lengthy exposures at temperatures higher than the physiological one will induce synthesis of heat shock proteins (HSP), western blot analysis of heat shock protein HSP-70 was performed. Western blotting experiments on control and exposed HaCaT cells did not reveal differences in HSP-70 expression, suggesting that exposed cells are not undergoing stress induced by heat effect (data not shown). Field intensity (B) measured with a calibrated Hall probe, is within −5% of center value inside the cylindrical exposure volume of 11 cm by 17 cm along the solenoid axis. The measured geomagnetic ambient field is 32 μT (vertical component) and 16 μT (horizontal component). Stray ambient ac fields are below 0.1 μT. An often overlooked fact related to solenoids is the presence of an almost homogeneous electric field oriented parallel to the magnetic field (Ellinger et al., 1989) unless adequate electric shielding is used (Stauffer et al., 1994). An approximate estimate of this field may be obtained from the voltage across the extremes of the inner of the six layers and its length. The corresponding field induced by capacity coupling in the culture medium will be in the order of 0.01 μV/cm (Foster and Schwan, 1995).

Sample Preparation

HaCaT cells were cultured in complete medium starting at a concentration of $5 \times 10^5\,mL^{-1}$ both for control and exposed samples. After 24 h cells were washed three times in PBS, air dried, and observed by IR-SNOM.

SNOM Imaging

The experiments here described were performed using an IR-spectroscopic version of scanning near-field optical microscopy (SNOM), in conjunction with a tunable free-electron laser source (FEL). Although any tunable IR laser should perform in an equivalent manner, the Vanderbilt Mark III FEL conveniently provides a broad tuning range of 1–10 μm, which is ideal for IR-SNOM application in biology (Coluzza et al., 1992; Pohl and Courjon, 1992). Details about the experimental design (Cricenti et al., 2002) are given (Figure 1). In our experimental setup, IR photons from the unfocused free-electron laser (FEL) beam are directed by a mirror onto the sample surface at an ∼75° angle to the surface normal. Reflected photons are detected through a narrow-point optical fiber tip mounted on a SNOM module, which also measures shear-force (topographic) images (Cricenti et al., 2002). During a scan, reflected IR light is picked up through the fiber and is detected by a photoconductive detector. Topographical and optical images were taken simultaneously, with the FEL illuminating the specimen over a broad area (∼1 mm spot diameter) and the SNOM probe collecting the reflected light. The shear-force signal is independently used to keep the tip-surface interaction force constant while taking SNOM images. In this mode, the data are collected at a present value

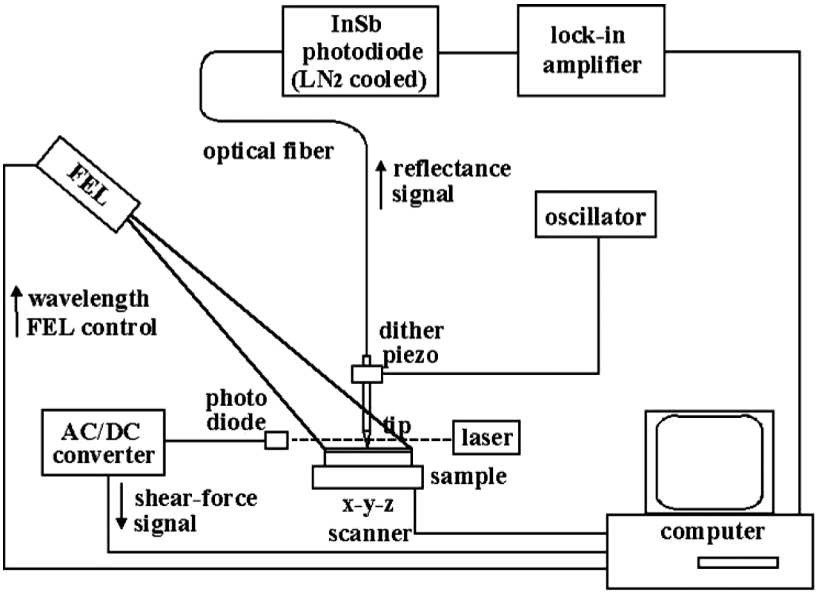

Figure 1. Schematic diagram of our experimental setup for IR-SNOM spectroscopy.

of shear force between sample and tip. This force should be kept constant when scanning the sample to have a constant shear force image (topography).

Although we collected images at several wavelengths, we will concentrate and show in this article only those at 3.04 and 6.45 μm for the following reason. It must be considered that in a complex system, such as a eukaryotic cell, there are many factors influencing the measurement. Although each chemical compound has its own vibrational mode, which is a sort of fingerprint, the different constituents of a cell give a spectra with complex and even overlapping absorption bands (Cricenti et al., 1998; Coluzza et al., 1992; Jamin et al., 1998, 2003).

Results and Discussion

Figure 2 shows topography and the corresponding infrared scanning near field optical microscopy of, respectively, unexposed (CTR) and 24 h exposed (EXP) HaCaT cells, analyzed at several different wavelengths, with a specific interest for the wavelengths $\lambda = 3.04\,\mu m$ and $\lambda = 6.45\,\mu m$.

The images shown in Fig. 2, at $\lambda = 6.45\,\mu m$ (Amide II peak), map the protein concentration and shows a quite homogeneous distribution covering almost the entire cell, according with their ubiquitary role.

It is interesting to observe the result obtained at $\lambda = 6.45\,\mu m$, a wavelength region typical of the reflection of proteins (Figure 2). In fact, a strong reflectivity signal could be clearly detected in the overall structure of exposed cells, confirming our previous finding concerning an increase of the adhesion molecule β4 integrin in the cell membrane of the exposed cells (Rieti et al., 2004). On the contrary, in control (unexposed) cells the reflectivity signal is less evident and at the same time more diffused on the whole sample.

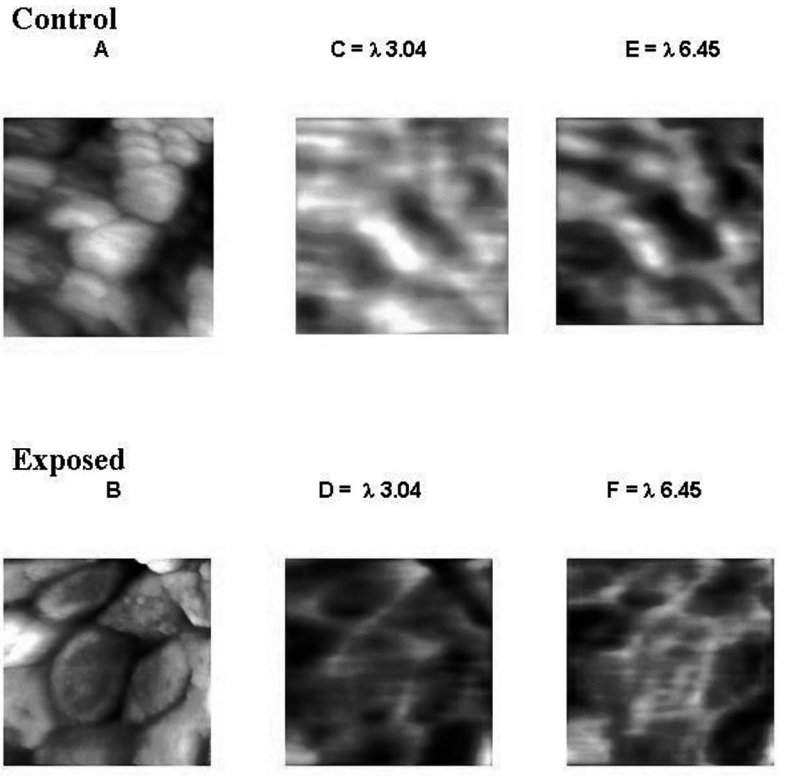

Figure 2. A and B:shear-force topography images of control and 24 h 50 Hz, 1 mT magnetic field exposed cells; C and D:IR-SNOM optical reflectivity images taken at a wavelength of 3.04 μm of control and 24 h 50 Hz, 1 mT magnetic field exposed cells; E and F:IR-SNOM images taken at a wavelength of 6.45 μm of control and 24 h 50 Hz, 1 mT magnetic field exposed cells. All images are $20 \times 20\,\mu m^2$.

It has been known from the literature that in a plasma membrane phospholipids organization can be affected by the plasma membrane proteins structure and composition, assuming that these proteins can induce a phase transition in the lipid organization from an ordered to a disordered domain. In the light of what we have pointed out, it is particularly interesting to find that, when we were analyzing the infrared absorption spectrum at $\lambda = 3.04\,\mu m$, the lipids reflectivity of unexposed cells was completely different from the one found for exposed HaCaT cells. This finding can be interpreted as a reorganization of the phospholipids mojety in the plasma membrane of the exposed cells, due to an increase in the synthesis of the adhesion membrane protein integrin.

Taken together, the reflectivity results at both $\lambda = 6.45\,\mu m$ and $\lambda = 3.04\,\mu m$ support one another in terms of an increase of newly synthesized protein in the exposed cells, jointly with a different phospholipids distribution in the exposed sample. This is in agreement with our previous results using fluorescence SNOM, that showed for HaCaT cells exposed to magnetic field, an increase of segregation in the cell membrane of the sulphur rich protein adhesion marker β4 integrin (Rieti et al., 2004).

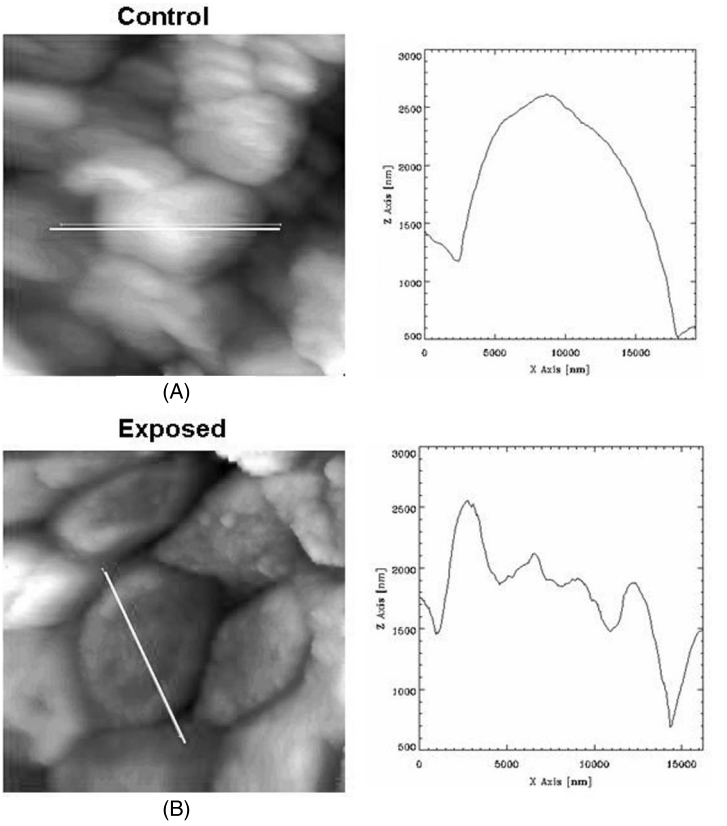

Figure 3. Shear force images ($20 \times 20\,\mu m^2$) of unexposed (A) and 50 Hz, 1 mT magnetic field exposed (B) HaCaT cells together with the corresponding A–B line profiles.

Furthermore, the effect of 50 Hz, 1 mT magnetic field on HaCaT cells is confirmed by the cross section of the cell topographic profile (Figure 3, A-B line), showing that the biochemical variations observed in exposed samples were also accompanied by a change in shape; in fact, while control cells showed an approximately rounded shape, exposed cells were more flattened. This is in agreement with previous AFM microscopy studies, where the HaCaT cells, exposed to the same magnetic field, underwent a modification in shape and morphology with an increase of the areas of adhesion between cells.

In conclusion, we have performed an IR wavelength-selective near-field measurements of a cell with the aim to localize the distribution of functional groups which are related to the biochemical variations suffered by cells exposed to a physical agent like the extremely low frequency (ELF) electric and magnetic field. Our results constitute another practical implementation of IR near-field microscopy for cell biological application.

Acknowledgments

This work as been partially supported by grant ISPESL B1/42/DIPIA/04 and B1/45/DIPIA/04.

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