Effects of Radiofrequency Radiation on Gene Expression: A Study of Gene Expressions of Human Keratinocytes From Different Origins

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Introduction

The answers to questions related to the biological effects of radiofrequency radiation exposure on organisms are pending. In bioelectromagnetic experiments, one of the main difficulties is that the results found in the literature, even those obtained with equivalent models, are poorly reproducible. This poor reproducibility may be due to the use of various biological material and exposure systems, varying exposure durations and, most of the time, varying frequencies. For instance, focusing on the impact of the electromagnetic field on gene expression, we found that the 8 most recent publications used microarrays based on 5 different models (mouse, rat, human, Drosophila, and chicken models); 8 different frequencies, from intermediary frequencies to radiofrequencies; and 3 data processing methods (no statistical tests, simple tests, and false discovery rate (FDR) statistical test) [Andocs et al., 2016; Fragopoulou et al., 2018; Habauzit et al., 2020; Kim et al., 2016; Lamkowski et al., 2018; Manta et al., 2017; Woelders et al., 2017; Yeh et al., 2015]. Together, the differences in these parameters make it difficult not only to compare individual experiments but also to replicate them.

For decades, evaluations of the potential effects of electromagnetic waves have focused on several pathways that could be involved in cancer promotion, such as DNA damage and oxidative stress pathways [Saliev et al., 2019; Vijayalaxmi and Prihoda, 2012]. The research

performed to date has intensively explored whole-genome gene expression modification under radiofrequency exposure [Leszczynski et al., 2012; Leszczynski, 2014]. In particular, genomic high-throughput approaches have enabled whole-genome gene expression to be screened under both exposure and control conditions, but such approaches have failed to produce clear agreements.

Our group has performed several microarray experiments to evaluate the effects of millimeter waves (MMWs) at approximately 60 GHz on gene expression [Habauzit et al., 2014; Le Quément et al., 2012; Soubere Mahamoud et al., 2016]. We have used microarray approaches with primary cultures of human keratinocytes as the main targets of MMWs. First, we have found that when MMWs are applied alone in athermic conditions and when microarrays are analyzed with a false discovery rate (FDR) filter, the MMWs have no effect on gene expression [Habauzit et al., 2014; Le Quément et al., 2012; Soubere Mahamoud et al., 2016]. However, when MMWs are applied at an incident power density (IPD) of 20 mW/cm² (the current International Commission on Non-Ionizing Radiation Protection (ICNIRP) upper limit for local exposure of the general public [Ahlbom et al., 1998]) in association with other stressors (heat or metabolic stresses, for instance), the results from the same analysis (with FDR-filtered data) reveal a slight effect of the MMWs on gene expression with 7 and 6 modified genes [Habauzit et al., 2014; Soubere Mahamoud et al., 2016]. Among the modified genes, 3 genes exhibit changes sufficiently reproducible for study: *ADAMTS6*, *IL7R*, and *NOG.* The aim of this study was to evaluate the universality of the expression modifications of these genes in other primary cultures of keratinocytes and a cell line.

Materials and methods

Cell cultures

Four cell types were used. The first cell type corresponded to a pool of primary human keratinocytes isolated from 3 neonatal foreskins and was called HEK_3N (Invitrogen, Saint-Aubin, France). The second cell type, called HEK_1N, consisted of primary human keratinocytes from a single donor of neonatal foreskin (Invitrogen, Saint-Aubin, France). The third primary culture (NHEK_3N) was derived from 3 pools of donated neonatal foreskin (Lonza, Levallois-Perret, France). All primary keratinocytes were cultured and exposed as previously described [Habauzit et al., 2014] and were used when they were between passages 4 and 9. Briefly, cells were cultured onto collagen IV-coated plates (Becton Dickinson, Franklin Lakes, NJ) in supplemented keratinocyte serum-free medium (SFM) (Gibco, Carlsbad, CA) with antibiotics (Invitrogen, Saint-Aubin, France). The derived keratinocyte cell line (HaCaT) was used and maintained in culture in Dulbecco's modified Eagle medium (Gibco/Life Technologies, Saint-Aubin, France) supplemented with 10% fetal calf serum (FCS), 1% antibiotics, and 1% L-glutamine as previously described [Le Quément et al., 2014]. The HaCaT cells were used when they were between passages 5 and 10.

Exposure system and experimental setup

The exposure conditions were optimized numerically to maximize the homogeneity of the specific absorption rate (SAR) distribution within the cell monolayer, as detailed previously [Zhadobov et al., 2012]. The IPD was determined numerically and then validated by thermal measurement. The average and peak SAR over the cell monolayer were 594 W/kg and 1233 W/kg, respectively, and corresponded in near-field conditions (2.5 cm between the horn antenna and the plate bottom) to an average IPD of 20 mW/cm². The exposure system and

conditions have been detailed previously [Zhadobov et al., 2012]. Briefly, the 2.10^5 cells, in one well of the 6-well plate, were placed in a MEMMERT UNE400 incubator (Memmert, Schwabach, Germany) adapted for electromagnetic exposure. The inside of the incubator was covered with absorbent material (ECCOSORB HR-1/2"-MB, Emerson & Cuming, Westerlo, Belgium) with a reflectivity below -20 dB around 60 GHz. This limits the maximum reflections to less than 1% in respect to the incident field. Due to the properties of MMWs, cell exposures were performed from the bottoms of the wells (Fig. S1). The cells were exposed in the corresponding culture medium supplemented with 10 mM HEPES for primary keratinocyte cultures and 4.6 mM HEPES for HaCaT cells for 3 h. For each experiment 4 ml of medium was used. Note that the volume does not influence either the SAR or the IPD [Orlacchio et al., 2019]. Two exposure conditions (unexposed (Sham) and MMW-exposed (Expo)) were used under the same cell culture conditions. All cells were exposed in the same incubator with the generator on or off. The experiments were replicated between 3 and 6 times.

RNA extraction and RT-QPCR analysis

RNA was extracted with a NucleoSpin RNA kit (Macherey-Nagel, Hoerdt, France) and then quantified by a NanoDrop 8000 spectrophotometer (NanoDrop Technology, Cambridge, UK). Five hundred nanograms of RNA was reverse-transcribed with an iScript kit (Bio-Rad, Hercules, CA) according to the supplier's instructions. All primers used are presented in Table 1. Two housekeeping genes (*TBP* and *GAPDH*) were used for normalization. Quantitative polymerase chain reaction (QPCR) was performed on a Bio-Rad CFX 384-well apparatus with SYBR Green Supermix (Bio-Rad, Hercules, CA). The results were analyzed using the ΔΔCT method.

Statistical analysis

Statistical analyses for the comparison of conditions were performed using a one-tailed Mann-Whitney test on GraphPad Prism software (GraphPad Software, San Diego, CA). A difference at *p-value* ≤ 0.05 was considered statistically significant.

Results

Effect of MMWs on gene expression in keratinocytes

ADAMTS6, *IL7*, and *NOG* gene expression in primary keratinocyte cultures was modified by treatment with growth factors, especially Interleukin-1 (IL1) and Epidermal Growth Factor (EGF). These data confirmed that the expression of these genes was sensitive enough to be modulated, as shown in Figure S2 (supplementary data). For the exposure experiments, 4 cellular models of the exact same cell type, human keratinocytes, were used. Among these cell models, 3 primary cultures were obtained from different donors, and one culture was based on a keratinocyte-derived cell line (HaCaT). Although the cell sources were different, the basal expression of *ADAMTS6*, *IL7R*, and *NOG* was similar in the four keratinocyte models (data not shown). Figure 1 shows the variations in *ADAMTS6*, *IL7R*, and *NOG* expression levels. Three different expression profiles were observed after MMW exposure. The first profile indicated that *ADAMTS6* expression was downregulated in HEK_3N cells, with a fold change of 0.5 with associated *p-value* of 0.057 (Fig. 1A). The second profile did not show any treatment effects, as illustrated by lack of differences in *ADAMTS6* expression levels in the sham and expo cells (similarly, no differences were found for *IL7R* and *NOG* expression; Fig. 1B-C) in the HEK_1N cells. The third *ADAMTS6* profile was characterized by upregulated gene expression in the expo NHEK_3N primary culture cells and the HaCaT cells with associated *p-*

value of 0.1, findings that corresponded to data previously obtained. Together, these three profiles indicate that each biological material shows a specific sensitivity, even when the exposure treatment and conditions are exactly the same. This finding was obtained regardless of whether *ADAMTS6* expression was downregulated, not affected, or upregulated. The same expression patterns were also observed for *IL7R* and *NOG* (Fig. 1B-C). Among these profiles, no significant differences were found.

Discussion

In our previous microarray experiments, 3 differentially expressed genes were identified, and one year later, the findings were confirmed by results from independent experiments based on the same cellular model [Habauzit et al., 2014]. These data indicated that MMWs significantly upregulate *ADAMTS6*, *ILR7*, and *NOG* expression. These previously published data [Habauzit et al., 2014] indicated a specific MMW effect independent of a heat-associated MMW response. In this study, we aimed to determine whether the gene regulation observed was specific to the type of cell used or whether it reflected a more general regulation that could be found regardless of the cell type. Therefore, experiments were replicated with three different primary cultures and one cell line. We first tested several growth factors to ensure that these genes could be modulated. These controls validated the RT-QPCR method used to evaluate the MMWs' effects on gene expression. Then, the duplicated experiments in the keratinocyte models showed 3 different expression patterns (downregulation, no effect, and upregulation after both treatments), suggesting that the specific sensitivity observed may depend more on the model used than on the general cell sensitivity to MMW exposure. This

observation raises two problems of paramount importance in the field of bioelectromagnetism: the reproducibility of the results and impact of the biological model.

Findings of bioelectromagnetic studies are difficult to compare because of the multiplicity of exposure systems, frequencies, treatment durations, models, and technologies required for biological assessment and statistical analysis. Attention is often concentrated on the variations in exposure conditions and wavelengths to explain differences between bioelectromagnetic studies. Consequently, the impact of the biological material is often underestimated. This problem and related problems linked to biological models have started to be illustrated by more general studies in published papers [Lai, 2018; Yakymenko et al., 2016].

The present study shows that the biological model exerts a strong influence on the data obtained, which may at least partly explain the heterogeneity of the reported results in the field. It is important to emphasize that these experiments were conducted at the same frequency (60.4 GHz), in the same exposure system, and for the same exposure duration. In addition, we used 4 cellular models that were all presumed to represent the same biological entity (human keratinocytes) and therefore were expected to react in a similar manner. Surprisingly, we found 3 different expression profiles despite using identical exposure conditions. The statistically significant effect in our first study (based on one primary culture pool) was not reproduced when the biological material was changed, although all the models were composed of keratinocytes (primary cultures sourced from one to three donors randomly obtained from suppliers and a cell line). We conclude that the biological material caused great variability in the cellular response. Two possibilities that are not mutually exclusive may explain such observations. First, it cannot be ruled out that the observed

variations were reflective of random fluctuations not related to the treatment. Considering their cost, most in vitro studies are repeated a limited number of times. In these cases, the limits of the statistical tests are reached, and false positives can sometimes emerge; for instance, a *p-value* of 0.05 indicates a 5% probability of the data representing a false positive. Second, the variability in the biological response to exposure could have been associated with the state or history of the biological material. It is well known that heterogeneous gene expression among cellular subclones can be the result of epigenetic modification or somatic mutation. Mutations are very rare events; in contrast, epigenetic modifications are common and relatively more sensitive to the environment. These modifications are essential for cell adaptation and responses to external variations, and they constitute a form of memory that, in turn, may influence how cells respond to environmental stimuli [Park et al., 2014; Veith et al., 2016]. Thus, it is possible that the different expression profiles observed reflect epigenetic modifications specific to the history/state of each cellular model that we used. If sensitivity to the electromagnetic field depends on epigenetic memory, then these results raise questions about inter-individual sensitivity to stimulation in the human population.

Although our past and present results are divergent, it is very likely that the observations were accurate each time. However, they reflected the situation at the moment of the experiment, which depended on the sensitivity and dynamism of the models used. We are aware that this study focused on only three genes and that the results may therefore not be representative of all expected results in bioelectromagnetism studies. Moreover, whether these results can be translated to other radiofrequencies and/or biological models, such as brains, remains unclear. This question is valid, and the uncertainties are illustrated by the many contradictory results described in the literature. Some publications in the field have started to introduce quality criteria [Simkó et al., 2016; Vijayalaxmi, 2016]. Among all the key

points considered in these publications, validation of the effects in other cell types (different models or equivalent models) should be the main criterion for the identification of clear and reproducible effects of radiofrequency exposure.

In conclusion, our data demonstrate that in the four keratinocyte cell types, 3 different expression patterns (down regulation, upregulation, and no effect) were observed, despite their exposure having been the same in all regards. Additional studies will be necessary to identify the molecular and cellular origins of such variability in exposure sensitivity.

For additional information, see Online Supplementary Materials on the publisher's website.

Legends

Table Legend

Table 1: List of primers used to validate the identified differentially expressed genes

Figure Legends

Fig. 1. *ADAMTS6* (A)*, IL7R* (B), and *NOG* (C) expression in different primary cultures and a cell line. The results are expressed as median, and the error bars represent the interquartile range. In Figure 1A, the replicate numbers are indicated in brackets. The associated *p-value* is indicated between each comparison.

Fig. S1. Illustration of the exposure system used for all the detailed experiments.

Fig. S2. Gene expression of *ADAMTS6* (A), *IL7R* (B), and *NOG* (C) under stimulation of Tumor Necrosis Factor (TNF-α, 50 ng/ml), Fibroblast Growth Factor (FGF, 10 ng/ml), Leukemia Inhibitory Factor (LIF, 10 ng/ml), Epidermal Growth Factor (EGF, 10 ng/ml), Interleukin-1 (IL-1, 50 ng/ml), and Transforming Growth Factor (TGF-β, 10 ng/ml). The results are the means from two independent experiments ± SEMs.

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Table 1: List of primers used to validate the identified differentially expressed genes

Gene Symbol	Forward	Reverse	RefSeq #
ADAMTS6	ACATCAATCCTCTTCCTCTGGG	TTCAAGTTCTGCAGTCGAGC	NM_197941.4
IL7R	GACGCATGTGAATTTATCCAGCAC	CATACATTGCTGCCGGTTGGAG	NM_002185.5
NOG	AAGCAGCGCCTAAGCAAGAAGC	AATGTCTGCGACCACAGCCACATC	NM_005450.6
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	NM_002046
TBP	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA	NM_003194

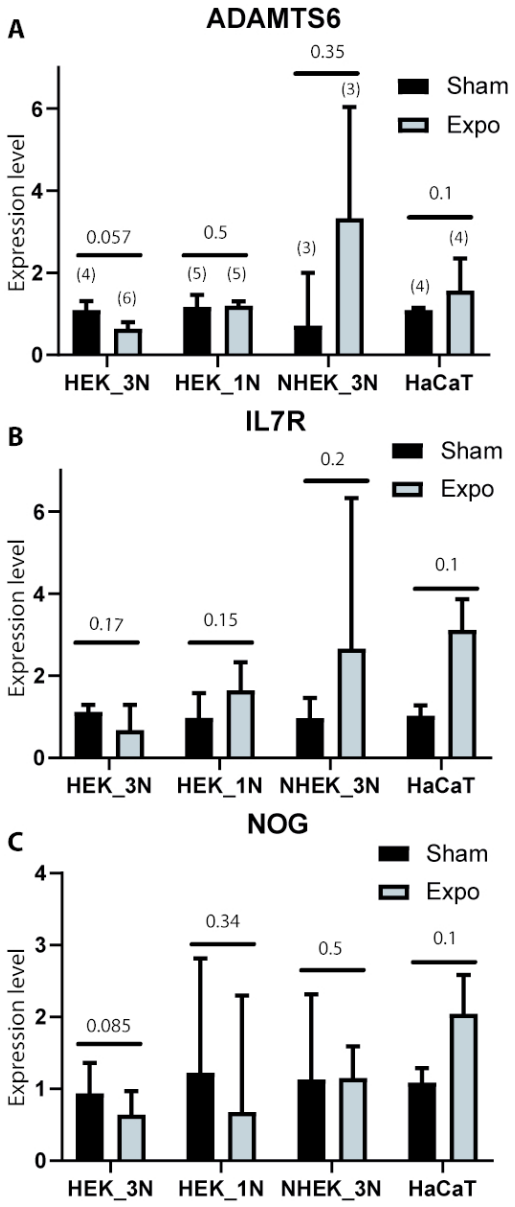

Fig. 1: ADAMTS6 (A), IL7R (B), and NOG (C) expression in different primary cultures and a cell line. The results are expressed as median, and the error bars represent the interquartile range. In Fig. 1A, the replicate numbers are indicated in brackets. The associated p-value is indicated between each comparison.