Differentiation of Human LAN-5 Neuroblastoma Cells by Electronically Trasmitted Retinoic Acid (RA)

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Abstract— We report the transfer of the activity of Retinoic acid (RA) by electronic means.

Retinoic acid were placed at room temperature on one coil attached to an oscillator (VEGA select 719), while LAN-5 neuroblastoma cells were placed on another coil and incubate under controlled condition. The oscillator was then turned on for 12 hrs a day for 5 days, after which cells were counted and morphology studied by contrast microscopy. In a control experiment the effect of the differentiating agent directly added to the cell culture could be observed by a decrease in cell growth, metabolic activity and the protrusion of neurite like structure typical of the differentiated cells. These preliminary results suggest that retinoic acid molecules emit signals that can be transferred to LAN-5 neuroblastoma cells by artificial physical means in a manner that seems related to the chemical structure of the source molecules.

These results suggest that RA molecules emit signals that can be transferred to LAN-5 cells by artificial physical means in a manner that seems specific to the source molecules.

1. INTRODUCTION

Previously Benveniste et al. suggests that the electromagnetic molecular signal (EMS) from pharmacological active molecules can be digitally recorded and replayed [1]. In the same work, they show that normal human neutrophils reacted to PMA transmitted via an electronic oscillator by reducing cytochrome c as though they had been directly exposed to PMA. The Benveniste finding of the electromagnetic molecular signal, was recently confirmed by the nobel laureate Luc Montagnier [2] in a work were he discovered a novel property of DNA, that is the capacity of some sequences to emit electromagnetic waves in resonance after excitation by the ambient electromagnetic background.

In the present study we electronically captured, and transmitted the specific electromagnetic signal (EMS) of Retinoic acid (a potent chemical molecule acting on human cells as a differentiating agent) to a biological system constituted by human neuroblastama cells line (LAN-5).

LAN-5 neuroblastoma cells represent one of the most common paediatric solid tumors originating from the sympathoadrenal lineage of neural crest. This tumor shows extremely different clinical phenotypes such as spontaneous regression on one hand and aggressive growth on the other hand. Undifferentiated neuroblastoma cell line (Lan-5) represent a good model to study neuronal differentiation induced by a variety of stimuli such as retinoic acid treatment.

2. MATERIAL AND METHODS

2.1. Cell Cultures

LAN-5 cells were grown in RPMI (Gibco Laboratories, Scotland) supplemented with 10% Fetal Calf Serum (Gibco Laboratories, Scotland) and antibiotics (110 IU/ml of penicillin and 0.1 mg/ml of streptomycin) at 37 ± 0.3°C, and 5% CO_2 as carbon source and sub-cultured twice a week at a 1 : 5 ratio. For every experiment, control and exposed cells were taken from the same flask.

2.2. Transmission Apparatus

For transmission experiments to cells, the input coil coupled to wave generator VEGA select 719 was operated at room temperature, while the output coil was placed in cell incubator. The source tube containing 5 µM RA and target coil containing LAN-5 cells. The electronic signal corresponding to RA was superimposed to both a 7 Hz sinusoidal frequency carrier modulated at 3 kHz.

The oscillator was then turned on for the 12 hrs a day for 5 days. During the experimental procedure, the various parameters such as power, voltage, capacitance and impedance remained constant.

2.3. Cellular Metabolic Activity and Proliferation by WST Assay

LAN-5 cells were exposed to the electronically transmitted RA EMS by Vega select 719. For each experiment LAN-5 cells were plated into 25 ml 4.2×5.2 cm base Corning flasks (2.0×10^5/ml cells in a total volume of 5 ml). The flasks were kept in the exposure system continuously for up to 5 days with or without RA-EMS. Cells were then counted and metabolism determined by WST-1 method. The experiment was repeated three times.

The quantification of LAN-5 metabolic activity, as an index of cellular proliferation, was performed by a colorimetric assay based on oxidation of tetrazolium salts (Cell Proliferation Reagent water-soluble tetrazolium salt (WST)-1; Roche Diagnostics, Basel, Switzerland). Cells were cultured for up to 5 days in a normal humidified incubator (control) or in the presence of the RA-EMS (exposed), and they were analysed by means of the formazan dye every 24 h. WST reagent diluted to 1 : 10 was added in the wells at 4 h, 1, 2, 3 and 6 days after plating, and then incubated for 2 h in humidified atmosphere (37 8°C, 5% CO_2). Quantification of the formazan dye produced was performed by absorbance measurement at 450 nm with a scanning multiwell spectrophotometer (Biotrack II; Amersham Biosciences, Little Chalfont, UK).

2.4. Immunofluorescence

For immunofluorescence staining the cells were grown in Labtek chamber slides. The cells were then washed with PBS with Ca/Mg and fixed with absolute ethanol for 5 minutes, then incubated with the specific monoclonal antibodies, anti-200 KDa neurofilaments (Sigma) appropiately diluted for 1 hour at room temperature. Cells were then washed three times with PBS and incubated with fluoresceinated anti-mouse IgGF(ab')2 fragment (Sigma), appropriately diluted for 1 hour at room temperature.

2.5. RT-PCR Analysis

Total RNA was extracted from cells using TRIzol Reagent (Life Technologies, Merelbeke, Belgium) according to the manufacturers' instructions. Typically 5–10 µg total RNA per 10 cm^2 dish of cell culture was obtained. Reverse transcription-polymerase chain reaction (RT-PCR) was used to evaluate relative mRNA levels of neurofilament protein (NF-200) in control and RA-EMRexposed LAN-5 cells. One microgram of total RNA was used to synthesize first-strand cDNA with random primers using 100 U of ImProm-II^{TM} RT-PCR kit (Promega, Madison, WI, USA) according to the manufacturer. The reaction was also carried out in the absence of reverse transcriptase (RT) to check for genomic DNA amplification. The NF-200 subunit-specific primers used for PCR were: 5'-aagtgaacacagatgctatgcg-3' 5'-ctgtcactccttccgtcacc-3' We used the 18 s as internal controls, because these genes are uniformly expressed during development. The subunit-specific primers used for PCR were: 5'-tttcggaactgaggccatgattaag-3' 5'-agtttcagctttgcaaccatactcc-3'. An aliquot (2 µL) of RT reaction was PCR-amplified in a final volume of 50 µL, by using 20 pmol of each primer, 200 µM of each dNTP, and 0.5 U of Taq DNA Polymerase (T. Aquaticus, Amersham-Pharmacia). PCR was carried out in a Bio-Rad I Cycle instrument. The thermo cycling conditions for each pair of primers were as follows: denaturation at 95°C for 3 min. followed by 30 cycles of denaturation at 95°C for 30 sec, annealing for 45 sec at 62°C, elongation at 72°C for 1 min and a final polymerization step at 72°C for 5 min for NF-200 and 20 cycles for 18 s. The amount of template and the number of amplification cycles were preliminarily optimized for each PCR reaction to avoid conditions of saturation. Aliquots (5 µL) of the reaction products were run on 1% agarose gels containing ethidium bromide (0.5 µg/ml) to mark and visualise the PCR products. Gels were then photographed under UV light with Versadoc (Bio-rad) instrument. These experiments were replicated three different times.

2.6. Statistical Analysis

Statistics was performed with Student's t-test with $P < 0.05$ as the minimum level of significance.

3. RESULTS

3.1. Electronically Transmitted RA Effect on LAN-5 Cell Metabolism

The cell growth rate was analyzed by the WST-1 both in LAN-5 cells as control (not exposed electronically transmitted RA) or exposed to the field. An inhibition in the cell metabolism in the electronically transmitted RA exposed was statistically ($p < 0.01$) significant after 5 days exposure (Fig. 1).

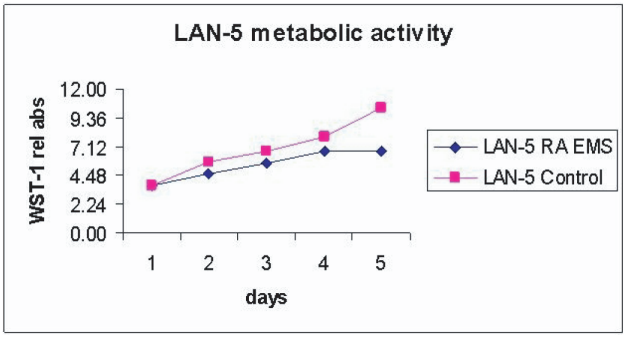

Figure 1: Cellular metabolic activity and proliferation by WST assay. LAN-5 metabolic activity by WST-1 analysis in presence (►) or absence (■) on RA-EMS.

3.2. Electronically Transmitted RA Effect on LAN-5 Cell Morphology

By phase contrast and scanning electron microscopy LAN-5 control cells appeared small, poligonal, without neurite-like structures. The exposure to electronically transmitted RA induced morphological changes toward a more neuronal phenotype: the cells were stretched out and rich of neurite-like structures with blebs, mimicking the same effect induced by retinoic acid treatment (Fig. 2).

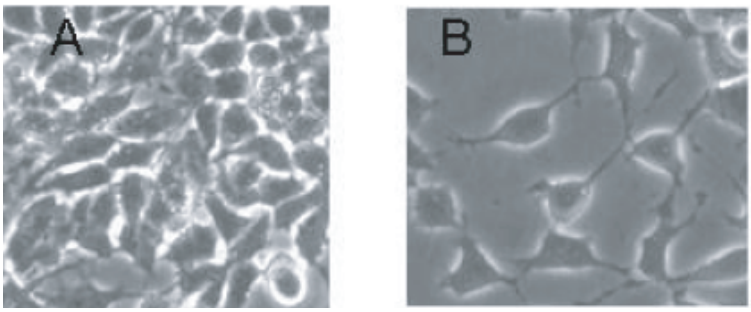

Figure 2: Electronically transmitted RA effect on LAN-5 cell morphology by contrast microscopy. Contrast microscopy of LAN-5 cells in absence (A) or presence of electronically transmitted RA on LAN-5 (B).

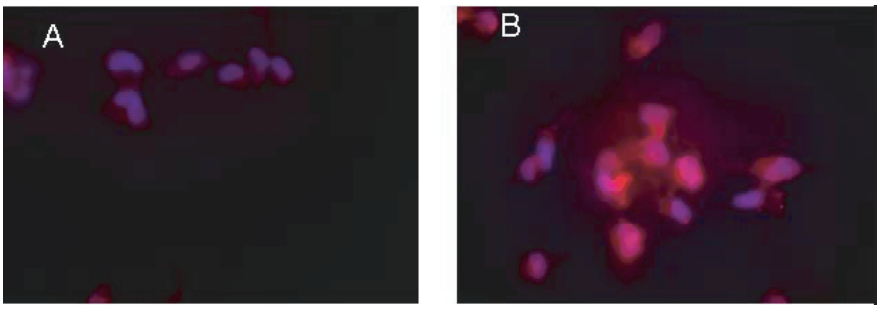

Figure 3: Electronically transmitted RA effect on LAN-5 cell by NF-200 indirect immunofluorescence. NF-200 indirect immunofluorescence of LAN-5 cells in absence (A) or presence of electronically transmitted RA on LAN-5 (B).

3.3. EMF Effect on Neurofilament Expression

Figure 3 shows the indirect immunofluorescent analysis of control and exposed Lan-5 cells with anti 200 neurofilaments. While control cell were little or not positive for NF 200 (CTR) the neurofilament protein become more fluorescent after exposure to the RA EMF (EXP). The same results were achieved by RT-PCR analysis for mRNA expression coding for NF-200. Fig. 4.

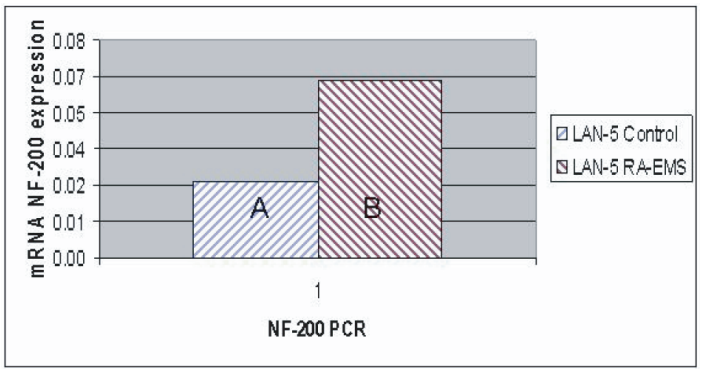

Figure 4: NF-200 mRNA RT PCR analysis on electronically transmitted RA effect on LAN-5 cell. Left bar (A) NF-200 mRNA RT PCR analysis on control LAN-5 cells, right bar (B) NF-200 mRNA RT PCR analysis on electronically transmitted RA effect on LAN-5 cell.

4. DISCUSSION

Low frequency electromagnetic fields at 50 or 60 Hz indeed are reported to stimulate nerve regeneration [3], alter gene transcription [4] and they may also play a synergistic role in cellular processes that are already activated, such as cell proliferation [5]. Despite an increasing number of publications demonstrate an effect of very low frequencies EM field on biological systems, other in vivo and in vitro studies suggest opposite results; in addition the possible interaction mechanism is not yet completely understood.

A possible mechanisms evoked to explain the mechanism of EM field action to biological system is involving Ca^{2+} transport across cell membrane, to trigger the signal transduction cascade [6].

Electromagnetic therapeutic potential can be seen in the proven efficacy of low-energy pulsed magnetic fields in non-union bone fracture healing, confirming that under certain conditions non-ionising electro-magnetic energy can influence physiological processes in organisms. Physiological paradigms for non-ionising radiation effects are required. Clues may be found in the mechanisms by which EM field interacts with cultured cells under controlled laboratory conditions and by correlating in vivo evidence with in vitro data [7]. Brain maturation depends on a sequence of postnatal events [8]. Brushart et al. [9] found that electrical stimulation at 20 Hz, promote motoneuron regeneration, confirming previous finding of the use of electric field for the orientation and growth of neurite [10].

12 hrs a day for five days exposure to RA-EMS field has significant effects on cells proliferation leading to a 30% inhibition of cell metabolism (Fig. 1). In addition with the impairment in cell metabolism RA-EMS electromagnetic field exposure, generated a morphological change as reported by the contrast microscopy study showed in Fig. 2.

In particular contrast microscopy analysis indirect immunofluorescence (Fig. 3) and RT-NF-200 PCR (Fig. 4) showed a more neuronal morphology characterized by the development of neuritic like processes in the exposed cells compared to control. Immunofluorescence using monoclonal antibodies for the major neurofilament proteins NF 200 unequivocally demonstrated an increase in synthesis and accumulation of these neuronal proteins in the RA-EMS exposed cells. Taken together all these data support an evident effect of the electronically transmitted retinoic acid (RA-EMS) electromagnetic field of driving neuroblastoma cells toward a neuronal differentiation, which resembles the effect determined by morphogens, such as retinoic acid in its chemical form.

ACKNOWLEDGMENT

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