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ETM study of electroporation influence on cell morphology in human malignant melanoma and human primary gingival fibroblast cells

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ABSTRACT

Objective: To estimate electroporation (EP) influence on malignant and normal cells. **Methods:** Two cell lines including human malignant melanoma (Me-45) and normal human gingival fibroblast (HGFs) were used. EP parameters were the following: 250, 1 000, 1 750, 2 500 V/cm; 50 μs by 5 impulses for every case. The viability of cells after EP was estimated by MTT assay. The ultrastructural analysis was observed by transmission electron microscope (Zeiss EM 900). **Results:** In the current study we observed the intracellular effect following EP on Me-45 and HGF cells. At the conditions applied, we did not observe any significant damage of mitochondrial activity in both cell lines treated by EP. Conversely, we showed that EP in some conditions can stimulate cells to proliferation. Some changes induced by EP were only visible in electron microscopy. In fibroblast cells we observed significant changes in lower parameters of EP (250 and 1 000 V/cm). After applying higher electric field intensities (2 500 V/cm) we detected many vacuoles, myelin-like bodies and swallowed endoplasmic reticulum. In melanoma cells such strong pathological modifications after EP were not observed, in comparison with control cells. The ultrastructure of both treated cell lines was changed according to the applied parameters of EP. **Conclusions:** We can claim that EP conditions are cell line dependent. In terms of the intracellular morphology, human fibroblasts are more sensitive to electric field as compared with melanoma cells. Optimal conditions should be determined for each cell line. Summarizing our study, we can conclude that EP is not an invasive method for human normal and malignant cells. This technique can be safely applied in chemotherapy for delivering drugs into tumor cells.

1. Introduction

Cell membranes are sensitive to physicochemical conditions, such as biochemical environment, temperature, mechanical stress, and electro-magnetic field. It was discovered that under diverse conditions plasma membrane of a cell looses its stability. Lipids in membrane change their conformation after application of electric field with proper parameters, thus they form non-stable hydrophilic pores[1-3]. This event was observed in 1958 for the first time. It was registered as unexplained, sudden increase of ionic conductivity through lipid membrane, and it is called electroporation (EP)[4-6]. The mechanism of EP is still not fully understood. Currently EP is used in cell culture as the purest available method of gene transfection[7]. First experiments with EP on animal models took place in the end of the 80s of the 20th century. Effectiveness of these attempts triggered research on the human organism, which began in the 90s[8-10], which resulted in the most important recent EP application-electrochemotherapy (ECT), accepted for treatment in EU in 2005. Electrochemotherapy uses the EP effect to facilitate transport of cytotoxic drugs inside malignant cell across cell membrane. Intentionally, the selected cytostatics were very poorly transported inside cells and demonstrated higher affinity to cancer cells. The experiments showed very significantly increased

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intracellular drug concentration limited to cancer cells exposed to the electric impulses. ECT can be applied locally in case of accessible cancers or tumors with superficial localization and internal organs. Impulses of the electric field in ECT are typically 100 μs long, with the intensity of 1 kV/cm. Cisplatin and bleomycin are used very often in this local treatment. Its effectiveness increases from 2-8 times in case of cisplatin, 500 times for bleomycin, and 3-5 times in case of actinomycin-D[11,12]. Current research shows very promising medical application of EP, such as immuno-genetherapy[10] and ECT with nanoimpulses[9].

Electrochemotherapy has been proved to be an excellent technique in melanoma treatment raising the complete response (CR) factor from 5% (standard chemotherapy) to 45% (ECT with bleomycin systemically), 77% (bleomycin locally), and 74% (cisplatin locally)[13-15]. Even new multidrug chemotherapy reaches CR not higher than 25% (for example Cisplatin CDDP +dacarbazin DITC, interleukin 2 (IL-2)+ interpheron 22b (INF-22b)[16]. Except for melanoma, ECT was mainly applied in basal breast cancer non-responsive to conventional treatment or various superficial neck and head cancers. CR in treatment of these cancers by ECT was about 55%[13]. Enhancing currently used therapies by EP open new perspectives in anticancer treatment, especially in case of multidrug resistance[5,9]. Application of combined therapy can be more efficient than usual chemotherapy and less radical than surgical intervention.

Although EP is effective in facilitating molecular transport, the intracellular effects of its application have not been sufficiently studied. The main intention of the presented study was examination of EP influence on cell morphology in two cell lines (normal and malignant) by electron transmission microscopy. The present paper shows unique studies of the EP influence on cell ultrastructure. The analysis of cell ultrastructure and determination of cells viability appeared proper techniques to study the EP impact.

2. Material and methods

2.1. Cell culture

We used human melanoma cells (Me-45) and human gingival fibroblasts (HGFs) from primary culture derived from a 30-year-old patient. Fibroblasts were isolated from soft tissue of gingival. The cells were isolated from the healthy gingival tissues according to the procedure described by Saczko *et al*[17]. Both cell lines were harvested in Dulbecco medium (Sigma) with 10% of FBS and addition of antibiotics. The cells were maintained in a humidified atmosphere at 37 ℃ in 5% CO_2.

2.2. Electroporation (EP)

The EP was carried out by use of ECM 830 (BTX) square-wave electroporator. At the high voltage mode, the electroporator provides electrical pulses with the magnitude 505-3 000 V/cm, 10-600 μs long, in the series of 1-99 impulses separated by the time interval of 100 ms-10 s. Different parameters of EP applied in our study were: 250, 1 000, 2 500, 3 250 and 4 000 V/cm; 50 ms in 5 impulses for every case. As electrodes we used two thin aluminum parallel plates, 4 mm apart. They were connected to the voltage generator and produced a uniform electric field in the cuvette (Cuvettes Plus 640, 800 μL). Cells in suspension were centrifuged for 5 min at 1 500 rpm and resuspended in the EP buffer with low electrical conductivity (10 mM phosphate, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4). Cells for examination by transmission electron microscopy were left for 5 min after pulsation, then cells were fixed for 30 min in 2.5% (vol/vol) glutaraldehyde and 0.1 M phosphate buffer (pH 7.4). Cells for MTT assay were left for 20 min with addition of 1600 μL DMEM after pulsation, then washed and centrifuged twice with DMEM.

2.3. MTT assay

The 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was used for the assessment of cell viability. After EP experiment, the cells were seeded into 96-well microculture plates (Nunc, NunclonTM Surface) at the concentration of 5×10^3 cells/well and incubated for 24 h at 37 ℃ and 5% CO_2. Then the medium of each well was replaced with 10 μL of 0.5 mg/mL MTT stock solution [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Saint Louis, MO; *In Vitro* Toxicology Assay] diluted in 90 μL PBS. After 2 h of incubation, isopropanol with 0.04 M HCl was added (100 μL/well). The absorbance was determined using a multiwell scanning spectrophotometer at 570 nm (Labsystem Multiscan MS type 352, Helsinki, Finland). Mitochondrial function was expressed as a percentage of viable cells under treatment relative to untreated control cells (the growth fraction of non-treated cells equals 1); the outstanding results not taken into account.

2.4. Transmission electron microscopy

Morphological assessment of cells after and before EP was examined by transmission electron microscope Zeiss EM 900. The cells were prepared according to the standard procedure. Cells were fixed for 30 min in 2.5% (vol/vol) glutaraldehyde and 0.1 M phosphate buffer (pH 7.4). After postfixation in 1% (wt/vol) osmium tetroxide, cells were dehydrated through a graded series of alcohol and propylene oxide and embedded in Epon.

2.5. Statistical analysis

The significance of the difference between mean values of different groups of cells was assessed by Student's *t*-test with *P*-value of $P=0.05$, to show the statistical significance. Results were analyzed by commercial software Statistica 9.0.

3. Results