THE EFFECT OF ELECTROPORATION ON THE ADHESIVENESS OF DIFFERENT EPITHELIAL CELL TYPES

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ABSTRACT

One of the many aspects of application of the electrical pulses on the cell membrane is the ability to form temporary pores. This phenomenon is known as electroporation.

The aim of this investigation is to study how electrical pulses influence on cell viability, cell-cell contact and integrate of the cells and reorganization of the actin cytoskeleton of different epithelial cell lines one of them is malignant.

It is shown that the changes in adhesiveness due to the applications of the electrical pulses with different electrical intensity are dependent on the type of malignancy of epithelial cells.

Key words. *electroporation, cell viability, actin cytoskeleton, cell morphology*

Intruduktion

The field intensity and duration of the applied electrical pulses of the electroporation (electropermeabilization) can either reversibly open nanoscale pores on the cell membrane after which the cell can survive, or irreversibly open the cell membrane, after which the cell dies [Davalos at all, 2005]. In cancer treatment, the reversible electroporation has been exploited to increase transport of chemotherapeutic drugs through the plasma membrane into the tumour cells. This process is called electrochemotherapy [Orlowski at all, 1999] and it is used for the treatment of the skin tumour lesions [Byrne at all, 2006]. Apart from the effect on cell membrane (to open nanoscaled pores), the applied external electric pulses demonstrate to be able to alter the cytoskeletal reorganization which affects the cell adhesion. For instance, changes in the cytoskeletal structure have been demonstrated during processes of electrofusion [Blangero at all, 1989] and electrotransfer [Rosazza at al, 2011]. Actin cytoskeletal redistribution has been reported in directional cell electromigration induced by electrical field [Titushkin at all, 2009] and in electroporation-based therapies [Kanthou at all, 2006]. Using adherent cells in the experimental model we can study the cells in their intact internal structure (cytoskeleton) and the results obtained in these cells are better comparable to real *in vivo* situations than the results from cell suspensions [Kanduser at all, 2009]. Comparing all data concerning actin cytoskeleton changes (how strong they can be and if they are reversible) in adherent cells induced by applied electrical pulses, it becomes visible that they depend mainly on the intensity of the applied field, electropulsation medium and cell type. In adherent cells, the basic actin-rich cell-extracellular matrix (ECM) ensembles are stress fibers, lamellipodia and filopodia, which play an important role in cell attachment and migration [Oelz at all, 2008]. Among the main features of the cancer cells are the breakdown of adherent connections (cell-cell contacts) and also the cytoskeleton organization (cell- ECM contacts) [Glukhova at all, 1995]. The change in the adhesive behaviour of cancer cells determines their modified morphology and migration behaviour and predetermines their invasive properties during all stages of tumourogenesis [Palovuori at all, 2003]. Thus, changing the cell's adhesion ability by electroporation it could be a very important prerequisite to inhibit cancer cells motility, invasion and metastasis.

Therefore, the aim of the study is to determinate the effect of the applied electrical pulses (100 -500 V/cm) on the adhesive behaviour of 3 epithelial cell lines. Two non-transformed epithelial cell lines MDCK and HaCaT are used as a control and A459 is selected as cell model for invasive and metastatic epithelial cancer cell line.

Materials and methods

Cells

Cell Line HaCaT Description

HaCaT in vitro spontaneously transformed keratinocytes from histologicaly normal human skin. The cell line was grown as monolyer (DMEM medium high glucose supplemented with 2mM L-glytamine 10% fetal calf serum and 1% antibiotic) at 37°C in an incubator with humid atmosphere and 5% CO_2. Cells were passage two times weekly by tripsinization (remove medium, rinse with 0.05% EDTA, add 0.05% EDTA solution and incubate for 10 min at 37°C. Take off EDTA, add fresh 0.05% trypsin/0.025% EDTA solution (final concentrations)) and let culture sit at 37°C until the cells detach (approximately 5 minutes). Add fresh medium, aspirate and dispense into new flasks). All mediums and chemicals are from Lonza, Belgium.

Cell Line MDCK Description

MDCK isolated from kidney of normal female adult Cocker Spaniel in 1958 by SH Madin and NB Darby (Madin Darby Canine Kidney). Culture Medium EMEM (EBSS) + 2mM Glutamine + 1% Non Essential Amino Acids (NEAA) + 10% Foetal Bovine Serum (FBS). Split using 0.25% trypsin/EDTA; 5% CO_2; 37°C. Cells attach firmly and require at least 2 PBS washes prior to addition of trypsin/EDTA.

Cell Line A549 Description

A549 cells are adenocarcinomic human alveolar basal epithelial cells. The cell line was grown as monolyer (DMEM medium high glucose supplemented with 2mM L-glytamine 10% fetal calf serum and 1% antibiotic) at 37°C in an incubator with humid atmosphere and 5% CO_2. Cells were passage two times weekly by tripsinization (remove medium, rinse with 0.05% EDTA, add 0.05% EDTA solution and incubate for 10 min at 37°C. Take off EDTA, add fresh 0.05% trypsin/0.025% EDTA solution (final concentrations)) and let culture sit at 37°C until the cells detach (approximately 5 minutes). Add fresh medium, aspirate and dispense into new flasks). All mediums and chemicals are from Lonza, Belgium.

MTT test

The viability of all cell lines was determined by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Applichem, Germany) dye-reduction assay. The MTT test (cytotoxic or therapeutic test) after the application of electric pulses was applied. To evaluate the statistical significance of the viability reduction of cells a comparison between exposed and control probes was performed by applying the two tailed paired Student`s test t-test, with p values lower than 0.05 considered statistically significant.

Electroporation

For electrotreatment a new electroporator (Chemopulse IV developed in the Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria) was used, which generates bipolar pulses. The instrument is equipped with a large voltage control in the limits of 100-2200V, simplified operations, locking against illegal manipulations, enhanced protection against electrical hazards, a battery supply, providing autonomy for more than 200 electroporations with one battery charge, and a recharging time for a depleted battery of less than 10 hours. The electrotreatment was done by 16 biphasic pulses, each of which lasted for 50+50μs with

20 ms pause between both phases and a pause between bipolar pulses of 880μs [Dotsinsky at all, 2012]. In each experiment, electrodes with an inter-electrode distance of 1cm were used. The intensity of applied electric fields was 100, 200 and 500V/cm. One hundred μl with $1.5x10^5$ cells were seeded 24h before electroporation.

Actin staining

Cells with density of $1.5x10^5$ cells/ml were cultivated on cover glasses (18/18 mm) placed in 6 well plates. After 24-hour incubation the cells were electroporated in a basal cell medium and were cultivated additionally for a period of 24 hours in full cell medium. After the incubation period, non-adhered cells were removed by triple rinsing with PBS, pH 7.4. The adhered cells were fixed with 1 ml 3% solution of PFA for 15 minutes at room temperature. The fixed cells were permeabilized using 1 ml 0.5% solution of Triton X-100 for 5 minutes and then incubated with 1 ml 1% solution of bovine serum albumin (BSA) for 15 minutes. The samples were washed three times with PBS, pH 7.4 and then incubated for 30 minutes at room temperature with BODIPY 558/568 phalloidin. Again, the samples were washed three times with PBS and once with distilled water, and then were installed on objective glasses by Mowiol. Preparations were analyzed using inverted fluorescent microscope (Leica DMI3000 B, Leica Microsystems GmbH, Germany) with object HCX PL FLUOTAR 63x/1.25 oil.

Results and Discussion

The experiments were carried out to clarify haw under the influence of electroporation different cell lines change there cell viability. A statistically significant reduction of cell viability was detected 24 h after pulse application.

On HaCaT cell line we found a correlation between cell viability and voltage of the applied pulses. About 20% reduction of cell viability was achieved at 500 V/cm. The cell viability after treatment with low or high voltage electric pulses on HaCaT keratynocytes cell line was given in Figure 1.

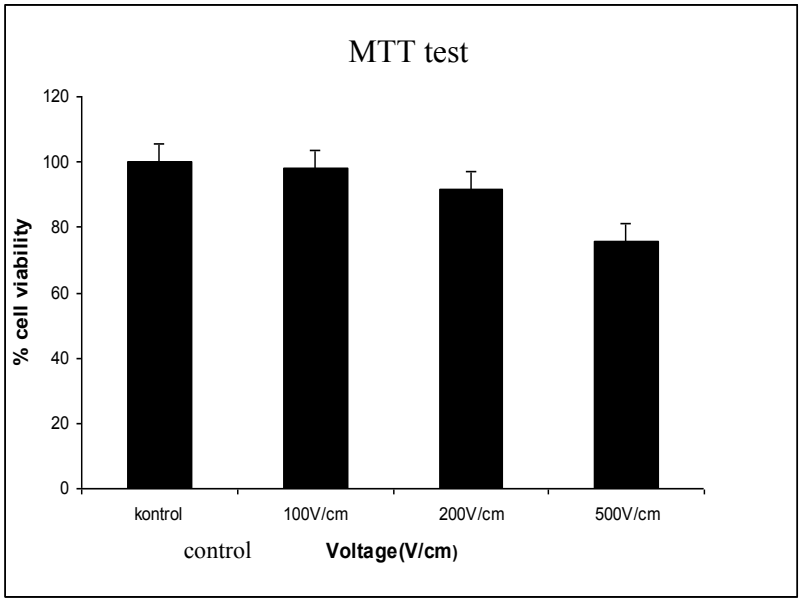

Figure 1. Viability of HaCaT keratinocytes after treatment with low and high voltage electric pulses Bars—SD (Standard Deviation); * $p < 0.05$ versus control and 500 V/cm significant difference between electroporated HaCaT cells.

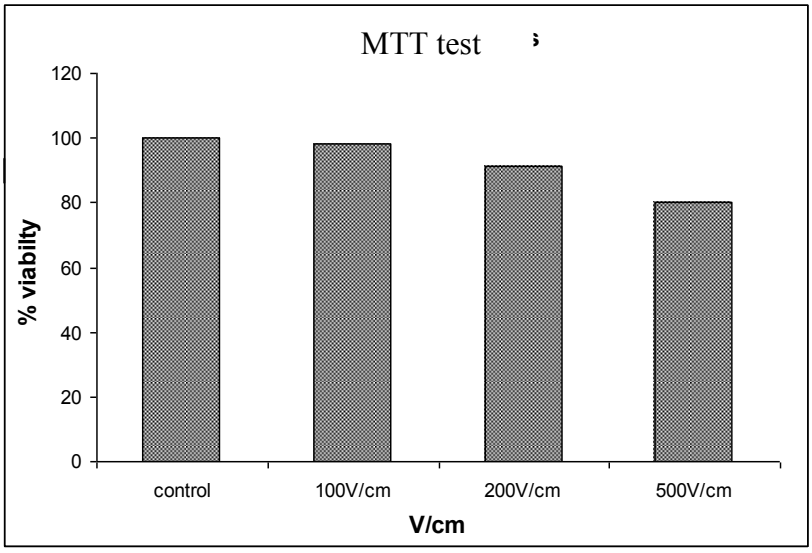

Figure 2. Viability of MDCK epithelial cells after treatment with low and high voltage electric pulses Bars—SD (Standard Deviation); * $p < 0.05$ versus control and 500V/cm significant difference between electroporated MDCK cells

On MDCK cell line we found also correlation between cell viability and voltage of the applied pulses again 20% reduction of cell viability was achieved at 500 V/cm.

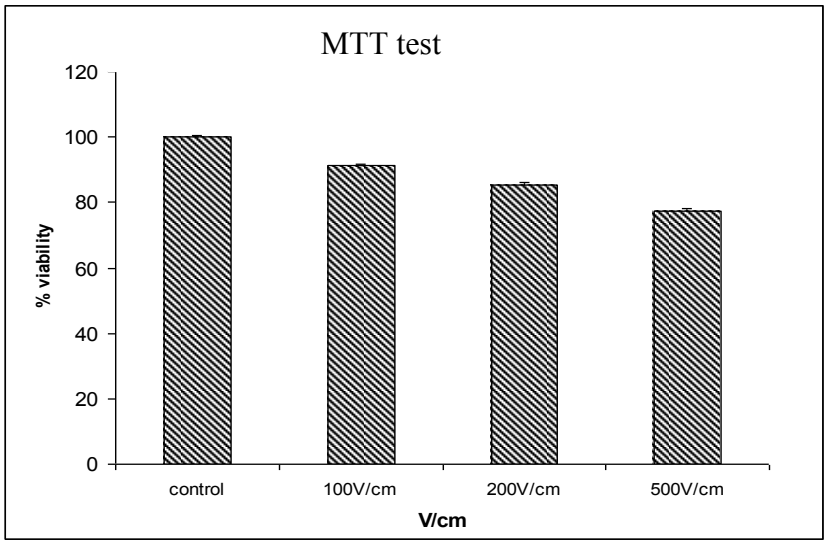

Figure 3. Viability of A594 cancer epithelial cells after treatment with low and high voltage electric pulses Bars—SD (Standard Deviation); * $p < 0.05$ versus control significantdifference between electroporated A594 .cells

On A549 cell line we found again correlation between cell viability and voltage of the applied electrical pulses near 20% reduction of cell viability was achieved at 500 V/cm.

The similar results concerning cell viability were achieved in two type cell lines, but the lowest reduction was observed in the malignant cell line A549.

That is why we examined the changes of the actin cytoskeleton after treatment with different electrical pulses.

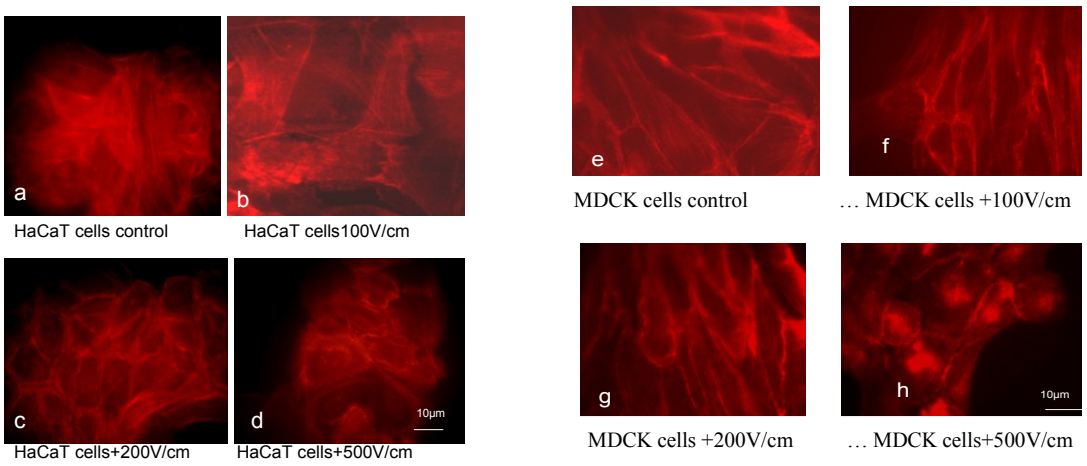

Figure 4. Actin staining of HaCaT and MDCK epithelial cell lines after treatment with low and high electric pulses. Bars 10 μm .

Cell adhesion is an important process and actin cytoskeleton plays a fundamental role in adhesion, migration and cell proliferation [Harkin at all, 1996]. The investigation of the influence of electrical pulses on the organization of actin cytoskeleton was conducted with one epithelial tumour cell line A549 and two nontransformed epithelial cell line MDCK and HaCaT. The organization of actin cytoskeleton was followed up to 24 hours after the electrotreatment in order to monitor how stable are the changes. Before the electroporation MDCK and HaCaT cells showed typical polygonal cell morphology with well pronounced intact actin filaments forming stress fibers (Figure 4a and 4e). After being electroporated with different intensities the reorganization of cytoskeleton actin filaments at low levels was observed. (Figure 4b-g). The actin filaments decreased in number and became thinner as the effect was amplified with the increasing of the field intensity. At 500V/cm the cytoskeleton was visible only in the cell periphery (Figure 4d and 4 h). In general, the results indicate a reduced strength of cell adhesion with the increase of the applied electrical pulses. The actin filaments were still organized in stress fibers in cells treated with low voltage electrical pulses 100 - 200 V/cm (Figure 4b,c,f,g). The recovery process of the intact actin cytoskeleton (the formation of stress fibers) 24 hours after electrotreatment seemed completed for the cells treated with 100 V/cm and 200V/cm (Figure 4 b, c) while the actin stress fibers in treated MDCK and HaCaT with 500V/cm remained pale and fine (Figure 4d and 4h). From the presented results it could be concluded that high voltage electroporation can lead to a fast disturbance of the actin filaments of MDCK and HaCaT cells and the process is intensity-dependent. Our findings are in agreement with other conducted experiments concerning the influence of the electroporation on the disturbance of the cytoskeleton structures of nontransformed cells as MDCK or HaCaT [Harkin at all, 1996]. We could suggest that the destabilization of actin cytoskeleton of the MDCK and HaCaT under the influence of high electrical pulses could lead to an additional positive effect for the electrochemotherapy - restriction of tumour expansion.

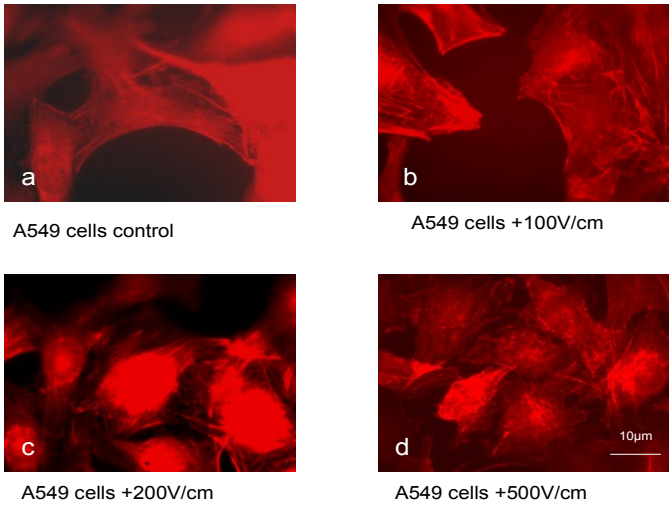

Figure 5. Actin staining of A549 cell epithelial cancer cells after treatment with low and high electric pulses. Bars 10 μm .

The electroporation with 100V/cm and 200V/cm of the invasive cell line A549 could not influence formation of well visible actin stress fibers (Figure 5 b,c,). Actin cytoskeleton at the control cells and after treatment with 100 and 200V/cm is typical organized (Figure 5a and 5b). The cells treated with 500V/cm have non-well-pronounced actin stress fibers very thin filaments organized near to membrane of the cells (Figure 5d). We demonstrated that in case of cancer cells electroporation with low voltage pulses did not cause a significant disturbance in actin cytoskeleton structures. Well visible actin stress fiberes as well as thin and dick actin filaments near to membrane could be seen in electroporated cancer cells predominantly on the 100V/cm and 200V/cm field intensity. It is known [Xiao et al, 2011] that the destruction of the cell cytoskeleton of tumor cells prevents them from necrosis and apoptosis when electrical field is applied. Based on the our results we could suggest that the application of high voltage electrical pulses to the transformed A549 lung epithelial cells could lead to their regression to a less or non-transformed cell phenotype since the consolidation of cell-cell contacts and actin organization leads to a reduction of cell motility and invasiveness. Also, in MDCK and HaCaT cells, an alteration in cell adhesiveness and cell phenotype is observed but the changes are related to the decreased of the number of actin filaments and amplified the ability of the electroporated cells to form stable normal stress fibers. It can be suggested that this tendency could lead to a formation of a cell phenotype with decreased cell motility and invasiveness.

In conclusion, our results point to the possibility that in some distinct stage of progression tumor epithelial cells (A549) gradually change their adhesive phenotype after electroporotion with low electrical pulses. Moreover studding the effect of the electroporation on cell adhesiveness and actin cytoskeleton, we found that cell adhesion and survival of MDCK and HaCaT cell lines are not affected significantly by the applied electroporation, but the electrotreatment of invasive cancer cell line A549 induces an increase in cell adhesion at 100 - 200 V/cm field intensities and decreased cell adhesion at 500V/cm like other normal epithelial cell lines. Actin cytoskeleton is differently influenced by electroporation in MDCK and HaCaT and cancer A 549 cells. In MDCK and HaCaT cells actin cytoskeleton is poorly disturbed, since in cancer cells treated with 100-200V/cm field intensities actin cytoskeleton is well presented with stress fibers.

We could claim that the electroporation of cells resulted in reducing the number of cells, reorganization of the cytoskeleton and changes in adhesive properties of the malignant cell line.

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