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Electroporation Transiently Decreases GJB2 (Connexin 26) Expression in B16/BL6 Melanoma Cell Line

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Abstract Connexins are proteins that form gap junctions. Perturbations in the cell membrane reportedly promote changes in the expression profile of connexins. Electroporation promotes destabilization by applying electrical pulses, and this procedure is used in electrochemotherapy and gene therapy, among others. This in vitro work aimed to study the interference of electroporation on the expression profile of GJB2 (Cx26 gene) and Connexin 26 in melanoma cell line B16/BL6. The techniques of immunocytochemistry, Western blot, and real-time PCR were used. After electroporation, cells showed a transient decrease in GJB2 mRNA. The immunostaining of Cx26 showed no noticeable change after electroporation at different time points. However, Western blot showed a significant reduction in Cx26 30 min after electroporation. Our results showed that electroporation interferes transiently in the expression of Connexin 26 in melanoma and are consistent with the idea that electroporation is a process of intense stress that promotes cell homeostatic imbalance and results in disruption of cell physiological processes such as transcription and translation.

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Introduction

Electroporation is a physical method used to improve delivery of nonpermeant molecules into cells. The technique was introduced by Neumann and Rosenheck (1972), and its mechanism has been studied for decades. It is used in several procedures, including gene transfer for various purposes. In clinical practice, electroporation has been used to potentiate the effects of cytotoxic drugs for cancer treatment, in a method called "electrochemotherapy" (ECT) (Mir et al. 2003). ECT permeabilizes the plasma membrane of tumor cells and allows anticancer drugs to enter directly into the cytoplasm and eventually kill tumor cells (Mir et al. 2003).

Electroporation by itself presents the potential to promote cell cycle arrest and apoptosis or Stand-Alone dependent pathway of p53 (Lepik et al. 2003), to promote increased HSPA family genes involved in cellular stress, and significant enrichment of genes involved in protein synthesis (Mlakar et al. 2009).

Cell membranes are composed of lipid bilayers serving as structures that isolate distinct functional environments that communicate by the flow of ions and signaling molecules; this communication is facilitated by connexins, proteins that are attached to cell membranes.

Connexins are a multigene family of gap junction proteins. These proteins contain highly conserved transmembrane domains, extracellular domains required for hemichannel or connexon pairing between adjacent cells, and a carboxyl-terminal region that serves as a docking platform for signaling complexes. Six connexin proteins

form the hemichannel or connexon with a central pore. Intercellular gap junctions form when hemichannels from adjacent cells dock onto one another. They are essential for the transduction of signals and can activate intracellular signaling by mediating transport of signaling molecules such as ATP (Zappitelli et al. 2014). The term connexin is abbreviated as Cx followed by weight in kDa, like Cx26 is a protein with 26 kDa, the gene that encode the protein receive the term gap junction (GJ) followed by the gene family (a, b, or c) and a sequencial number, such as GJb2.

Connexins are reportedly involved in carcinogenesis; neoplastic cells have shown alterations in either expression or localization of connexins, leading to deficient communication capacity. Connexins have been classified as conditional tumor suppressors, since their decreased expression may be involved in the initial steps of carcinogenesis, or their over-expression may facilitate invasion and metastasis in later stages of the disease (Cronier et al. 2009; Hervé et al. 2007; Ito et al. 2000; Leithe et al. 2006; Naus & Laird 2010).

The half-life of connexins can undergo drastic changes in different cell types. These variations can occur depending on the intracellular pH, temperature, and phosphorylation, among other causes. Specifically in the case of connexins, they are related to membrane proteins that form gap junctions, the fact that the cells are adhered to or not and possibly that disturbances and alterations in plasma membrane may interfere with the half-life and therefore the expression profile of these proteins (Hervé et al. 2007).

The phenomenon of electroporation promotes disturbance in the membrane after exposure to a specific electric field (Kanduser et al. 2006; Spugnini et al. 2007). However, this could promote alteration not only in lipids of the membrane: electroporation can induce changes in proteins attached to the cell membrane, like Cx26 and proteins that act in signal transduction.

B16/BL6 cells are a well-known cell lineage that is derived from an aggressive murine melanoma. When inoculated into mice, it grows not only in the subcutaneous region, but also causes spontaneous metastasis. B16/BL6 cells show a basal expression of Cx26 (Ishiguro et al. 1996; Ezumi et al. 2008). The study reported here aimed to assess whether the disruption of the melanoma cell membrane, promoted by electroporation, interferes with the expression profile of Connexin 26 in the B16/BL6 melanoma cell line.

Materials and Methods

Experimental Design

Samples of the melanoma cell line B16/BL6 were exposed to an electric field to promote specific reversible electroporation (8 pulses of 800 V/cm of width, 100 μs duration at a frequency of 1 Hz). After applying the electric field, the samples were evaluated at different times after exposure. The time points evaluated were as follows: zero (t0), where the samples were prepared for analysis immediately after application of the field, half-hour after field application (t0.5), 1 h (t1), 3 h (t3), and 5 h (t5), always relative hours after application of field. In samples t0.5, t1, t3, and t5, the cells were maintained in standard condition for cell culture (37 °C, 5 % CO_2, and atmospheric humidity) until the time of sample preparation. In the control group, samples were exposed only to the electrode without applying the electric field in the same manner as in other groups. Each group was examined in duplicate and at three different times.

Culture of Melanoma Cell Lineage B16/BL6

B16/BL6 melanoma cells (supplied frozen by Prof. Dr. Hiroshi Yamasaki—School of Science, Kwansei Gakuin University, Uegahara, Nishinomiya, Japan) were cultured in RPMI-1640, supplemented with penicillin (50 UI/mL), streptomycin (50 mg/mL), and L-glutamine (2 mmol/L) with added 10 % fetal calf serum in Petri dishes of 100 mm under standard conditions 37 °C, 5 % CO_2, atmospheric moisture. The cells remained in the culture until confluence minimum of approximately 80 %, when they were subjected to the electric field.

Electroporation of Samples

The electrode used was Petri pulser BTX (Fisher Scientific—Cat. Number 45-0130) with 100 mm diameter, arranged in 24 cupper plates with 3.5 mm of distance between them. The plates were attached to PVC support and connected by copper wires. Upon reaching a minimum of approximately 80 % confluence, the monolayer of adherent melanoma cells in RPMI-1640 medium were exposed to 8 electric pulses of 280 V amplitude, 100 μs in duration and frequency of 1 Hz. BTX apparatus 830 was used to generate the electrical pulses (Fisher Scientific). After the application of electrical pulses, cells were placed again under standard conditions (37 °C, 5 % CO_2, and atmospheric humidity) until the specific time for sample preparation. Control experiments using eletroporation of ethidium bromide were performed to verify the level of permeabilization and cell viability (data not shown). Methodology was adapted from Heller et al. 2005.

Cx26 Immunofluorescence

To visualize, the Cx26, 2×10^5 cells of melanoma cell lines were plated on acrylic coverslips of 35 mm and

cultured in RPMI-1640, supplemented with penicillin (50 UI/mL), streptomycin (50 mg/mL), and L-glutamine (2 mmol/L) by adding 10 % fetal calf serum. After exposure to the electric field, cells were fixed in 2 % paraformaldehyde for 10 min at room temperature and permeabilized in Triton X-100 (Sigma-Aldrich) 0.1 %, diluted in 5 % milk for 30 min at room temperature. The cells were washed three times in PBS after each of the cases. They were then incubated overnight at 4 °C with primary antibodies against Cx26 (polyclonal antibody diluted in Triton X-100 0.1 % in milk at 5 % final concentration of 1:20, Invitrogen). Then, secondary antibodies (Alexa 488, Invitrogen), anti-mouse immunoglobulins, and biotinylated phalloidin (Sigma) were applied, diluted in skim milk with 5 % Triton X-100 0.1 % at a concentration of 1:100. This was followed by conjugation with streptavidin-fluorescein (FITC) to obtain fluorescence. To avoid depletion of fluorescence, the preparations were mounted in a specific medium (Vectashield + DAPI; Interchim). The material was photographed under a microscope Nikon E-800 system equipped with fluorescence and digital photomicrograph.

Western Blot

After exposure to the electric field, cells in culture were washed with PBS and scraped from Petri dishes using cell SRAP (Corning) in the respective times appointed for the study. Later they were homogenized in a buffer containing 60 mM Tris–HCl, pH 6.8, 2 % SDS, 12 % glycerol, dithiothreitol, and 0.1 M phenylmethylsulfonyl fluoride (PMSF). Protein concentration in homogenate was determined using the Bio ®-Rad kit and reading in a spectrophotometer at 595 nm. Because of the effect of cell detachment that electroporation can promote, the solution of PBS used for washing was also centrifuged and the pellets of cells formed was also used for protein extraction, thus avoiding reduction in levels. The proteins were applied to polyacrylamide gel at 12 % sodium dodecyl sulfate (SDS) at 100 V until the bromophenol blue fall at the end of the gel. The proteins were then transferred to polyvinylidene difluoride membranes (PDVF) for 50 min at 100 V. The membranes were blocked in 5 % skim milk for 2 h at room temperature. We used antibodies to Cx26 diluted (1:200—Invitrogen) in PBS solution containing 5 % skim milk and applied to the membranes. The preparation was incubated overnight under mild agitation, at 8 °C. After incubation with anti-rabbit immunoglobulin conjugated with peroxidase, the reaction was revealed with a solution containing diaminobenzidine (Sigma), nickel sulfate, and hydrogen peroxide. Relative quantification of the intensity of staining was performed in Image Master System (Amersham Pharmacia Biotech).

Real-Time PCR

After exposure to the electric field, the cells were scraped from Petri dishes using cell SRAP (Corning) in the respective times appointed for the study. After scraping, the cells were placed in plastic tubes and centrifuged at 1500 rpm and 4 °C for 10 min. After centrifuging, the culture medium was discarded and the RNA extraction with RNAspin mini kit (GE Healthcare) started. The extracted RNA was subjected to reverse transcription using the enzyme Superscript II reverse transcriptase and oligo dT primers. This was followed by Real-Time PCR technique using the TaqMan system according to the manufacturer's instructions (Applied Biosystems). We used certificates primers and probes from Applied Biosystems—Foster City, CA with the following ID: Cx26 (GJB2) (Mm00433643_s1) and 18S (4319413E). Data were analyzed using relative expression reported by Livak (2001) using the 18S gene as internal control of the reaction and the control group as a parameter for normalization.

Results

Immunostaining of Cx26 in Cell Lines B16/BL6 Submitted to Electroporation

We used the immunofluorescence technique to detect presence and location of Cx26 in B16/BL6 cells, according to the times after exposure to the electric field. In T0 cells (Fig. 1a), it is possible to note the abundant presence of cytoplasmic Cx26. These cells showed the same pattern of labeling like the cells in the control treatment (data not shown). In the T0.5 cells (Fig. 1b), marking of Cx26 protein was very weak and only cytoplasmic as well as in the treatment control and T0. In T1 (Fig. 1c), it is possible to note the wide presence of Cx26 in the cytoplasm more intensely than in cells T0.5, and in T3 and T5 cells (Fig. 1d, e), it is possible to observe the intense immunostaining of Cx26 into the cells in a way very similar to T0 treatment.

Quantification of Connexin 26 Expression by Western Blot B16/BL6 Cell Lines Submitted to Electroporation

After identifying changes in the pattern of labeling the cells analyzed by immunofluorescence, we performed the quantification of Cx26 levels by Western blot (Fig. 2). The densitometry analysis of the bands shows that T0.5 cells have lower values than control cells (p value: 0.0045). The T0 cells showed values statistically equal to control treatment. After 1 h of exposure to the electric field levels of Cx26, proteins are resettled and no statistically significant differences were found in T1, T3, and T5 (p value: 0.3612).

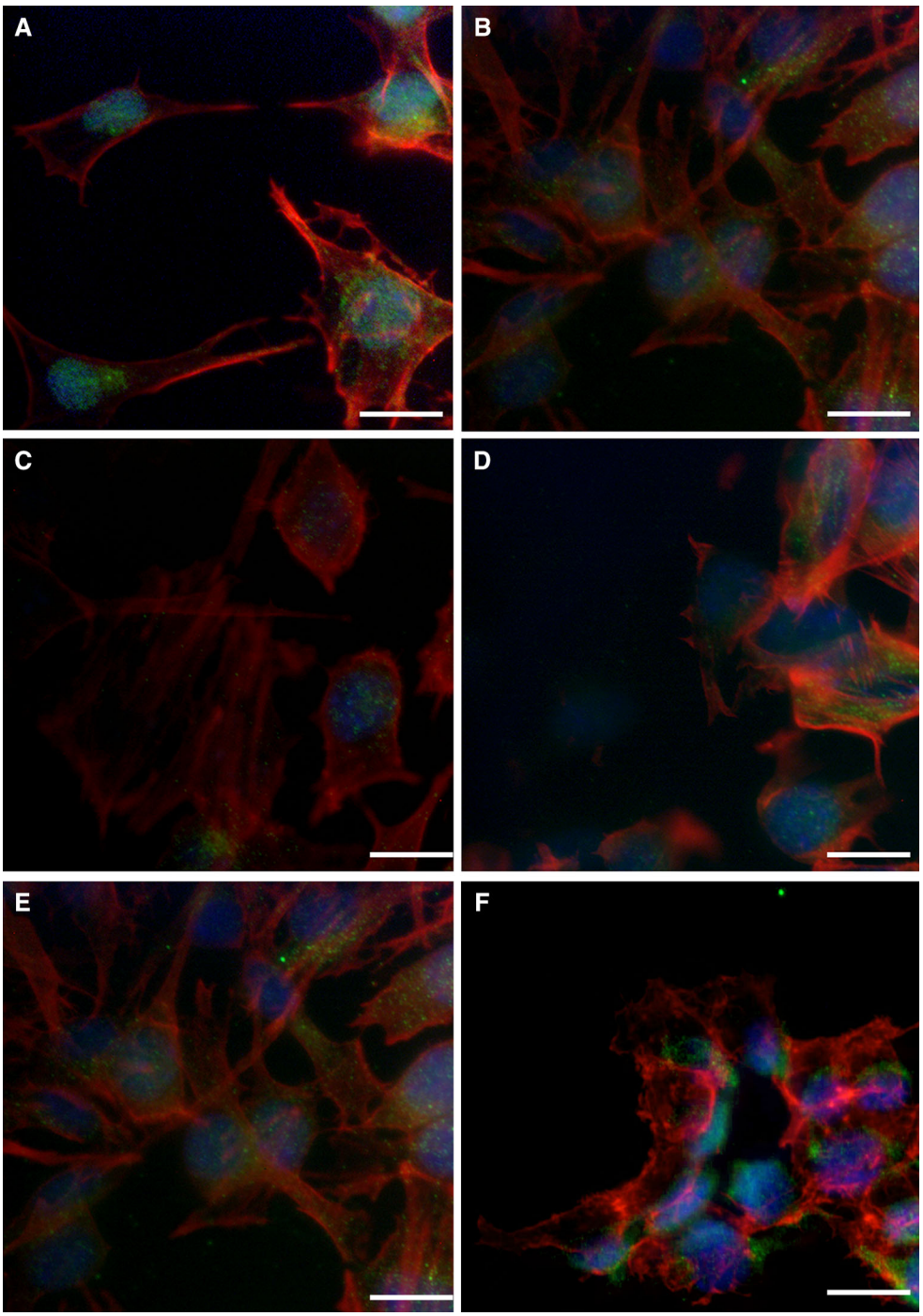

Fig. 1 Immunofluorescence for Cx26 (*green*) in B16/BL6 cells exposed to electric field and analyzed at different times after exposure. T0 (analyzed immediately after exposure—**a**); T0.5 (half-hour—**b**); T1 (1 h—**c**); T3 (3 h—**d**), and T5 (5 h—1**e**). The location of Cx26 in all cells is cytoplasmic, is clear the decrease of labeling in T0.5 cells. Nuclei are stained in *blue* and cytoskeleton in *red*. *Scale bar* 20 μm (Color figure online)

GJB2 mRNA Transcription in B16/BL6 Melanoma Cells Undergoing Electroporation Using the Technique of Real-Time PCR

Knowing that T0.05 cells showed a smaller amount of Cx26 protein, we sought to understand whether this decrease was due to decrease of expression of Cx26 gene or post-transcriptional mechanisms. To answer this question we analyzed the expression of Cx26 gene by Real-Time PCR technique, and our results showed that T0.5 cells have lower levels of Cx26 gene when compared with the control cells (p value : 0.0351). The T1 cells

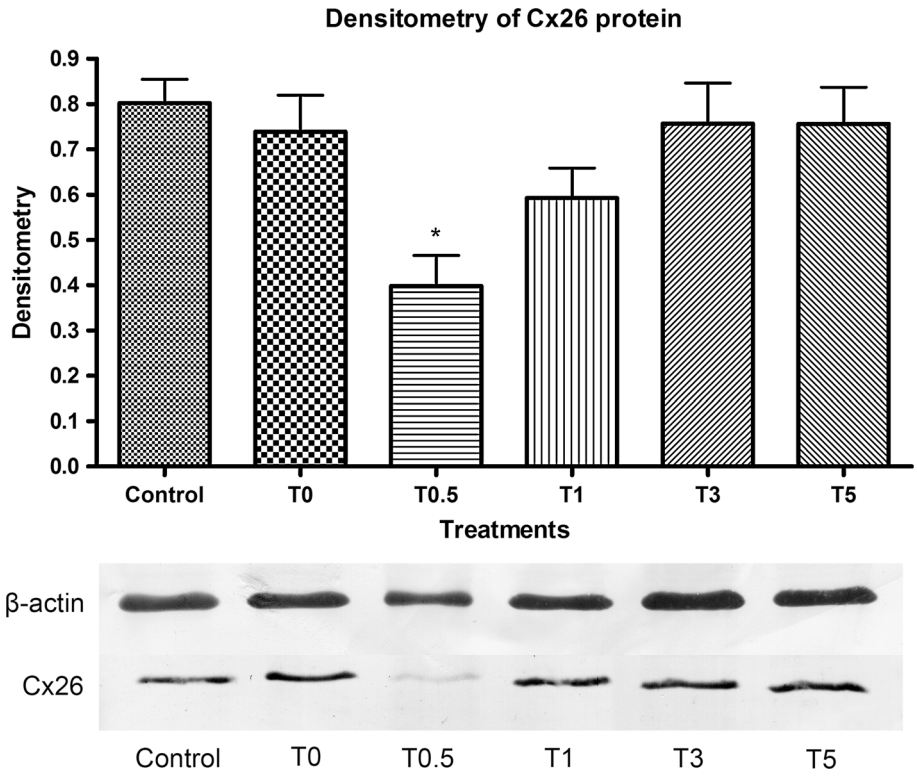

Fig. 2 Quantification of Cx26 levels in B16/BL6 cells by densitometry of bands obtained in Western blot technique. Control (not exposed to electric field); T0 (analyzed immediately after exposure); T0.5 (half-hour); T1 (1 h); T3 (3 h), and T5 (5 h). The data were normalized using B-actin like endogenous control. The T0.5 treatment showed a *lower level* of protein when compared with the control treatment. The *asterisk* represents statistically significant differences when compared to the control treatment

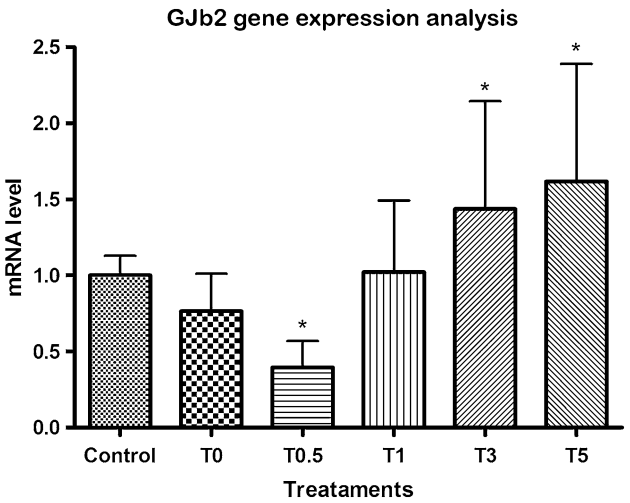

Fig. 3 GJB2 mRNA levels at different times after electroporation observed through the technique of real-time PCR in melanoma cell line B16/BL6. Control (not exposed to electric field); T0 (analyzed immediately after exposure); T0.5 (half-hour); T1 (1 h); T3 (3 h), and T5 (5 h). We can observe that the group whose mRNA was extracted half-hour after applying the electric field (T0.5) was the only one where it has been found a reduction with a significant difference when compared to the other groups. The treatments T3 and T5 showed increase of GJB2 mRNA levels. The data were normalized using B-actin gene like endogenous control, and mRNA of cells not exposed to electric field were used to compare treatments. The *asterisks* represent statistically significant differences when compared to the control

showed levels of expression similar to control, while T3 and T5 cells showed higher levels than the control cells (p value: 0.0211) (Fig. 3).

Discussion

This study was conducted to determine the effect of electroporation on gene expression, focusing on the expression of GJB2 and its encoded protein, Connexin 26. Initially, immunostaining of Cx26 was performed in order to verify the subcellular localization of this protein in B16/BL6 melanoma cells. The immunofluorescence technique revealed that Cx26 was present in the cytoplasm and not in the membrane, its normal localization. However, considering that B16/BL6 melanomas are malignant tumor cells, this cannot be considered an unusual finding (Ezumi et al. 2008).

The quantitative evaluation of Connexin 26 was obtained by Western blot technique. The results for the strains of B16/BL6 melanoma show a significant decrease in protein level of Cx26 in time T0.5, or 30 min after electroporation. We also observed the same pattern of decrease in mRNA levels. However, this decrease proved reversible, since from 1 h after electroporation, T1 the values of GJB2 mRNA are equal of Control and T3 and T5 treatments increased significantly, this enhance of expres-

sion was responsible of return Cx26 levels in T1, T3, and T5. A recent study on the effect of electroporation in melanoma cell lines showed low expression of a larger number of genes involved in protein synthesis (Mlakar et al. 2009). This is consistent with our results, since a decrease of GJB2 mRNA was obtained. The same study showed that genes involved in cancer development showed little difference in expression after electroporation process and HSPA family of genes that are involved in cellular response to stress, presented overexpressed (Mlakar et al. 2009).

It is known that each protein of the cell has a defined intracellular stability and quantity, as a result of opposing processes of synthesis and degradation. The regulation of protein expression in fact depends on fine-tuning the balance between various processes such as gene transcription, RNA processing, protein synthesis and layout, post-translational modifications, transport to the cell surface, anchoring to the cytoskeleton, regulation of endocytosis, and controlled degradation of the protein (de Sousa Abreu et al. 2009; Hervé et al. 2007). Subtle changes in one or more of these steps may involve changes in the regulatory level.

Our results are consistent with the idea that electroporation is a process of intense stress that promotes cell homeostatic imbalance and results in disruption of cell physiological processes such as transcription and translation, however transiently. The processes involved in this imbalance need to be further studied.

In conclusion, the effect of reversible electroporation, according to the protocol used in this work, promotes transient decreases in the levels of Cx26, both in mRNA and in protein. These reductions are possibly the product of a disturbance in cellular homeostasis, in turn, promoted by the stress of the process.

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