Extent of Cell Electrofusion In Vitro and In Vivo Is Cell Line Dependent

SONATA ŠALOMSKAITĖ-DAVALGIENĖ1,2, KAROLINA ĖPURNIENĖ1, SAULIUS ŠATKAUSKAS1, MINDAUGAS S. VENSLAUSKAS1 and LLUIS M. MIR3,4

1Department of Biology, Vytautas Magnus University, Kaunas;
2Department of Histology and Embryology, Kaunas University of Medicine, Kaunas, Lithuania;
3CNRS UMR 8121, Institute Gustave-Roussy, Villejuif;
4Univ Paris-Sud UMR 8121, France

Abstract. Background: Electric pulses delivered to cells that are in close contact may induce cell fusion, by a process termed electrofusion. Recently it has been shown that electrofusion in tumours in vivo depends on tumour type. The aim of this work was to examine the differences in electrofusion in various cell lines, both in vivo and in vitro. Materials and Methods: LPB, B16F10 and DC-3F cells in vitro and LLC tumours in vivo were exposed to various electric pulses. The number of fused cells was then evaluated. Results: Cell electropermeabilization was confirmed to be a necessary but non-exclusive condition to obtain a high level of cell electrofusion. The extent of electrofusion depends both on the degree of permeabilization and cell type. Conclusion: It was observed that metastatic tumour cells easily electrofuse, suggesting that cell type-specific membrane properties and/or secretion of proteases determine the extent of electrofusion.

The delivery of appropriate short and intense electrical pulses (EP) to living cells, either in suspension or in tissue, results in a transient and reversible alteration of their cell membrane (1). The phenomenon is known as cell electropermeabilization or cell electroporation. This technique is now widely used in order to transfect bacterial, fungal, plant and animal cells, as well as to introduce a variety of compounds into cells: small metabolites, dyes, drugs, peptides, enzymes and others (1-5). Similarly, electroporation allows enhanced delivery of some anticancer drugs into the tumour cells to be achieved both in vitro and in vivo (6, 7). The cytotoxic effect of the anticancer drugs is thus significantly enhanced. This technique has resulted in a new tumour treatment termed electrochemotherapy. Electro-chemotherapy combines administration of an anticancer drug (for example, bleomycin or cisplatin) and local delivery of EP at the tumour site. This therapy has proven to be very effective in the treatment of cutaneous tumour nodules in vivo and in cancer patients (6-13).

Another rapidly emerging application of electro-permeabilization is electrogenetherapy (14). The method (like electrochemotherapy) is based on electroporation of cells in tissues, which then allows the uptake of plasmid DNA in greater amounts.

Electrical pulses can also result in cell fusion in vitro (1, 15-17) as well as in vivo (18-20). Membrane fusion is a phenomenon often observed in nature, e.g. during secretion (fusion between the plasma membrane and the membrane of cell internal vesicles), during the formation of secondary lysosomes, during egg fertilization (21), viral infection (22) and tissue repair (23). In vitro cell-cell fusion can be induced artificially by adding fusogenic agents (e.g. polyethylene glycol, Sendai virus) or by subjecting cells to EP. The latter approach for cell-cell fusion is termed electrofusion (20, 24-26). In vitro electrofusion is a simple method to obtain hybridomas (27-29). The fusion product (hybridoma cell) has the properties of both parents, offering the possibility of producing monoclonal antibodies against bacteria, viruses and natural substances (26). Grasso et al. and Heller achieved ex vivo and in vivo electrofusion of human cells to rabbit cornea (18, 19), Mekid and Mir reported the occurrence of cell fusion within some type of tumours in vivo after they were exposed to permeabilizing EP (20). These authors exposed two types of tumours to the EP and found that cells were electrofused only in B16 but not in LPB tumours.

In this study, it is shown that cells of those tumour models that have a high propensity to electrofuse in vitro maintain this propensity in vivo in tumours following EP treatment.

Correspondence to: Lluís M. Mir, UMR 8121 CNRS, Institute Gustave-Roussy, 39 rue C. Desmoulins, 94805 Villejuif Cédex, France. Tel: +33 142114793, Fax: +33 142115245, e-mail: luis.mir@igr.fr

Key Words: Electroporation, electropermeabilization, electrofusion, electrochemotherapy, tumour treatment.

0250-7005/2009 $2.00+.40
This study thus suggests that intrinsic cell membrane and/or extracellular matrix properties play an important role in defining cell propensity for electrofusion.

Materials and Methods

Cell treatment in vitro. LPB sarcoma, B16F10 melanoma and Chinese hamster lung fibroblast DC-3F cells (obtained from Dr. J. Belehradek Jr) were used in the *in vitro* experiments. Cells were grown in minimum essential medium (MEM; GIBCO, UK) supplemented with foetal bovine serum (FBS) (8%) and antibiotics (penicillin and streptomycin). Cells were seeded at 3-5x10^4 cells per cm² on glass coverslips in 35 mm Petri dishes (Falcon) supplemented with 2 mL of the culture medium and allowed to grow in an incubator at 37°C in a humidified atmosphere containing 5% CO₂. One day later when the cells reached confluence of 70%, the culture medium was discarded and EP were delivered to the cells on coverslides in Spinnder essential medium (S-MEM; GIBCO). Two parallel plate stainless steel electrodes, 8 mm apart, were placed onto the monolayers and 8 EP of 100 μs duration and either 800, 1,000, or 1,200 V/cm were applied at a 1 Hz repetition frequency using a Cliniporator™ (IGEA, Italy). After the delivery of EP cells were incubated for 5 min at room temperature. Thereafter they were placed in MEM. Two hours later, cells were examined using a light microscope (Leica DMIL 520804, Germany). Experiments were repeated twice for each pulse strength. From each experiment, four images were taken in the electroporated area and one image from the non-electroporated area. For the analysis, the total number of fused cells in each image was counted.

Treatment of tumours in vivo. C57Bl/6 female mice were used in the experiments. They were kept at constant room temperature (22°C) with a natural day/night light cycle. All experiments were performed in accordance with guidelines for animal health and safety. The Lewis lung carcinoma (LLC) tumour model was used in the study. To obtain solid tumours, the tumour tissue was removed from mice bearing LLC and minced with a pair of scissors after addition of 5 mL of physiological saline. Subsequently, 0.2 mL of the obtained solution was injected subcutaneously. Six to ten days after tumour cell transplantation, mice were randomly divided into experimental groups and their tumours exposed to a specific treatment. When performing electrochemotherapy, bleomycin (BLM) at a dose of 10 mg/kg was injected intravenously 3-4 min before the delivery of the EP to the tumour. Electric pulses were delivered by two parallel stainless steel plate electrodes placed at the opposite margins of the tumour. The tumours were then pulsed with 8 square-wave pulses of 900, 1,300 or 1,500 V/cm pulse strength and of 0.1 or 1 ms pulse duration that were generated by a pulse generator made locally. Pulse repetition frequency was 1 Hz.

Histological analysis of tumours. The tumours from all experimental groups were removed for histological processing 3 days after the treatment. Specimens were fixed immediately in 4% buffered neutral formalin for 24-48 hours. The tissue was processed for dehydration in rising concentrations (50, 60, 70, 80, 96 and 100%) of alcohol. Then the tumour tissue was immersed in xylene (two washes of 1.5 hours each) followed by its immersion in liquid paraffin for 1.5 hours initially and then for 24 hours. After its embedding in the paraffin, tumour tissue was cut into 4 μm slices using a Reichert-Jung microtome (Germany). The slices were stained with haematoxylin and eosin by a standard method. Each experimental condition included 3-4 tumours. For each tumour, 5 randomly chosen slices were analysed. The tumour slices were examined under a light microscope (PZO, Poland) equipped with an automatic digital camera (Canon, Japan). Images were analysed using the Image Tool program (Dental Diagnostic Science, San Antonio, TX, USA). The percentage of nuclei in giant cells was determined with respect to the total number of nuclei in the slice.

Statistical analysis. The results are expressed as the percentage of fused cells±SEM. Statistical differences between experimental groups were calculated using Student’s *t*-test. Statistically significant difference was accepted when *p*<0.05.

Results

In vitro electrofusion. B16F10, LPB and DC-3F cells were treated with EP at the electric field strengths of 800, 1,000 and 1,200 V/cm. The extent of fusion was determined in electrically treated and non-treated areas. In the areas not exposed to EP, only separate cells with one single nucleus per cell were observed. No spontaneously fused cell was observed in the three tested cell lines. In the areas exposed to EP, large fused cells with several nuclei in the middle of the cell, surrounded by a thin sheet of cytoplasm were frequently detected. The morphology of the fused cells is presented in Figure 1.

Various levels of cell fusion were detected in the areas subjected to EP. Interestingly, the levels of cell fusion were clearly dependent on the cell line. Almost the same high percentage (64–69%) of fusion was found in the B16F10 cell line, irrespectively of the applied field strength (Figure 2). On the contrary, fusion in LPB and DC-3F cells was significantly lower (*p*<0.001) than the fusion in B16F10 cells. Although the fusion level in LPB and DC-3F clearly depended on the field intensity, it never reached that obtained in the B16F10 cell line. At 1,200 V/cm, the extent of B16F10 cell fusion was about 2 times higher than that of the LPB and DC-3F cells. At 1,000 V/cm and 800 V/cm, it was 3 and 9 times higher than LPB cell fusion and 2 and 5 times higher than DC-3F cell fusion, respectively (Figure 2). The possibility that a lower extent of LPB and DC-3F cell fusion was obtained because of incomplete cell electroporation can be excluded, since these cells were nearly 100% electroporated under the same condition at 1,200 V/cm. Indeed, to evaluate the percentage of cell permeabilization, Lucifer Yellow, a useful cell permeabilization marker was used (3). Exposure of B16, LPB or DC3F cells to 8 EP (1,200 V/cm, 100 ms) in the presence of Lucifer Yellow revealed that almost all cells in the area between the electrodes were actually permeabilized (data not shown).

In vivo electrofusion. Electrofusion was also detected *in vivo* in LLC tumours after electrochemotherapy treatment using 10 mg/kg BLM dose. Indeed, the electropermeabilized cells
were slowly killed by the BLM and both the percentage of permeabilized and of fused cells was analysed. No fused cells were found in control tumours (Figure 3A). Treatment with BLM alone does not induce cell fusion either \textit{in vitro} or \textit{in vivo} (data not shown).

In electrochemotherapy treatment, two different pulse strengths (1,300 and 1,500 V/cm) and two durations (0.1 and 1 ms) of electric pulses were applied (Figure 3B). Under the lowest permeabilization condition (1,300 V/cm, 0.1 ms) the extent of fusion was 18%. Under the stronger permeabilization conditions (1,500 V/cm, 1 ms), the extent of fusion increased to 33% (Figure 3A).

Permeabilization levels in the tumours were assessed using BLM, which has been shown to be a suitable marker for cell permeabilization \textit{in vitro} and \textit{in vivo} (30). Indeed, using appropriate concentrations and injection route, BLM is effective only in electropermeabilized cells (that can uptake the drug), while it does not provoke cell death of the cells that were not permeabilized by the EP. Thus the extent of necrosis can reveal the extent of cell electropermeabilization, despite the presence of some spontaneous necrosis, particularly in the rapidly growing experimental tumours transplanted in mice. In fact, a low level (about 13%) of cell death was actually found in control tumours (Figure 3B). In the electrochemotherapy groups exposed to 1,300 V/cm pulses of 0.1 and 1 ms of duration, the extent of necrosis was 73 and 90% respectively. Electrochemotherapy with 1,500 V/cm pulses induced 100% of cell death at both 0.1 and 1 ms pulse durations (Figure 3B).
Discussion

In this study, the extent of cell electrofusion was analyzed in vivo in the LLC tumour model and in vitro using the B16 melanoma cell line and two fibroblastic cell lines (the murine LPB and the Chinese hamster DC-3F cells) under the influence of EP. Cells were exposed to EP of various parameters known to provoke different levels of cell permeabilization. It was shown that cell electropermeabilization is necessary to produce cell electrofusion, both in vitro and in vivo. The results also give clues as to the factor(s) that could either restrict or facilitate the fusion of the electropermeabilized cells.

Indeed, these results are consistent with previous observations of Mekid and Mir (20) performed in vivo in the LLC tumour model and in vitro using B16 transplanted melanomas and LPB fibrosarcomas. The results reported here reveal cell electrofusion in vivo in Lewis lung carcinomas (Figure 3), which resembles the electrofusion obtained by Mekid and Mir (20) in melanoma B16. In contrast to melanoma B16, these authors observed almost no cell fusion in vivo in LPB fibrosarcoma. In the present study, these observations were extended by evaluating the levels of cell electrofusion in B16, LPB and DC-3F cells grown in monolayer. Even though all three cell types were easily electropermeabilized, only the B16 cells fused to a great extent, even at moderate electric field conditions (800 V/cm, 0.1 ms). Increasing the electric field strength did not increase the percentage of fused B16 cells. LPB and DC-3F cells displayed very low electrofusion at 800 V/cm but this percentage increased when stronger EP were used. Nevertheless, even at the strongest EP used (1,200 V/cm), when all cells of each line were permeabilized, the level of LPB or DC3F cell electrofusion was much lower (p<0.001) with respect to that of the B16 cells (Figure 2). The differences found here in vitro between the B16 and the LPB cells are thus comparable to the differences found in vivo in the Mekid and Mir study (20). Notably, both DC-3F and LPB cell lines, contrary to B16, are fibroblastic cells.

In all the conditions tested (that is either in vivo or in vitro, with the LLC, B16, LPB or DC-3F cells), it was shown that electropermeabilization is a condition necessary for electrofusion. In this study, it was found that the degree of permeabilization is always larger than that of electrofusion, in vitro and in vivo (Figure 3). While levels of in vitro permeabilization were not very different between the cell lines, the extent of electrofusion showed an important dependence on the cell line (Figure 2). In vivo, it was also shown that the extent of electrofusion increased with the strength and duration of EP (Figure 3) which correlates with an increase in tissue electropermeabilization (measured by the cell death induced by the BLM entering the electropermeabilized cells). Taken together, it can be concluded that (i) the extent of electrofusion is dependent on the degree of permeabilization; (ii) both in vitro and in vivo, cell electropermeabilization is necessary but not sufficient alone to induce cell electrofusion; and (iii) strain differences (e.g. between LPB and B16 cells) have a major impact on the extent of cell electrofusion.

The present study showed that cell electrofusion is also possible in LLC tumours in vivo, which supports the hypothesis of Mekid and Mir that metastatic tumours tend to electrofuse more easily than non-metastatic tumours. Indeed, both LLC and B16 tumours are highly metastatic and electrofuse to a high extent. In contrast, no cell electrofusion was found in non-metastatic LPB tumours (20). It is known that metastatic tumours release proteases into the environment that facilitates tumour cell migration into surrounding tissues or blood circulation. In turn, these proteases may induce changes in extracellular matrix or integrity of cell membrane proteins that might render cells more susceptible for cell fusion after stimulation of the tissue with permeabilizing EP.
Although no cell electrofusion was found in non-metastatic LPB tumours in vivo (20), LPB cells do fuse if permeabilizing EP are delivered to LPB cell an monolayer. Nevertheless, in comparison to B16 cells, the extent of LPB electrofusion was significantly lower (p<0.001) at the three pulse strengths tested. Why LPB cells were able to electrofuse in vitro but not in vivo remains to be explained. One possible explanation is that cell line differences in membrane lipid and protein composition might account for the cell fusion differences after cell electroporation. On the other hand, extracellular matrix of LPB cells could undergo some specific changes and/or reduction due to trypsinization of the LPB cultured cells during the consecutive passages. As a matter of fact, it has already been shown that cell treatment by trypsin facilitates plated CHO cell fusion in vitro (15). Such a hypothesis would be in full agreement with the analysis of the observations made in vivo and discussed here above.

In conclusion, this study revealed that cell electrofusion in vivo is possible in LLC tumours, which, like B16 melanoma, are also highly metastatic. Cell electropermeabilization is a condition necessary but not sufficient alone to obtain cell electrofusion, both in vitro and in vivo. In vitro findings showing that the extent of electrofusion in B16 cells is significantly larger than in LPB cells support the hypothesis that cells from metastatic tumours have greater propensity for cell electrofusion. Altogether, the data suggest that cell type-specific membrane properties and/or secretion of proteases determine the extent of fusion of electropermeabilized cells.

Acknowledgements
We are grateful to Professor N. Pauziene the head of the Laboratory of Electron Microscopy, Institute of Anatomy (KMU) for the contribution to the histological analysis and for technical advice. This work was supported by grants of CNRS, Institute Gustave-Roussy (IGR), European Commission (Cliniporator QLK3-1999-00484), Marie Curie Fellowship European Programme (5th PCRA, contract QLG-A-1999-50406) and the grants of Lithuanian State Science and Studies Foundation (K-C 17/2).

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Received October 24, 2008
Revised May 19, 2009
Accepted June 3, 2009