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First explanations for differences in electrotransfection efficiency *in vitro* and *in vivo* using spheroid model

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ABSTRACT

Electro-gene-therapy is a promising technique for cancer treatment. However, knowledge about mechanism of gene transfer with electric field in tumor is limited. Whereas *in vitro* electrotransfection is efficient, gene expression in tumoral cells *in vivo* is weak. To determine reasons for this difference and unravel gene transfer mechanisms, we propose to use multicellular tumor spheroid as a tridimensional model *ex vivo*. Comparison of efficiency between cell in suspension and cells in spheroid allow highlighting fundamental differences. For classical electrical conditions (consisting in 10 pulses of 500 V/cm, 5 ms, 1 Hz), suspension cells present a transfection rate of 23.75% ± 2.450 SEM. In the same conditions on spheroid, although plasmid DNA coding GFP interact with half of electrically permeabilized cells, less than 1% of cells are expressing the transgene. First answers to *in vivo* electrotransfection failure are given: cell mortality due to electric field is responsible of this low transfection rate, as tridimensional and multicellular structure that prevents DNA passage. These results show that spheroid is reproducing *in vivo* situation. Validation of spheroid as a relevant model for electrotransfection study opens *ex vivo* optimization possibility before *in vivo* assay.

1. Introduction

Anti-tumoral treatments are a public health priority since several years. Various therapies exist but often show weaknesses in efficacy or safety. This explains the need for further development of new anti-tumoral strategies. Among them, gene therapy shows promises to implement new approaches to cancer treatment. A large variety of methods for gene delivery, including viral, chemical and physical vectors have been developed for the past twenty years. Electroporation is a physical technique first developed to transfer cytotoxic drugs in tumor (Gothelf et al., 2003; Mir et al., 1998). It consists in the transient permeabilization of the plasma membrane following electric field application (Golzio et al., 2010; Rols et al., 1992). Electropermeabilization enables poorly permeant drugs such as bleomycin and cisplatin to efficiently enter cells and tissues. This process, called electro-chemo-therapy (ECT), allows controlling the amount of drug that enter the cells as well as targeting of the entry: only cells and tissues present in between the electrodes will be permeabilized. Therefore, the method is both efficient and safe. Indeed, it is strictly localized at the site of the tumor and the dose of cytotoxic drugs injected into the tumors is lower than the ones used in classical chemotherapy protocols. All together, these properties limit side effects and enhance drug activity. Nowadays ECT is used as a palliative treatment of cutaneous and subcutaneous cancer. In 2010, more than 1000 patients have been treated over Europe (www.cliniporator.com, Mir et al., 2003).

Besides ECT, another modality (electro-gene therapy – EGT), has been developed for more than 10 years and clinical trials are underway (Daud et al., 2008; Heller and Heller, 2006; Heller et al., 1996; Titomirov et al., 1991). As for ECT, EGT offers a gain in safety and in efficacy as gene is injected directly in the tissues where electric field pulses are applied. However, gene expression efficiency highly depends on tissues. Indeed, electrotransfection is not effective in all cell types *in vivo*. Transfection in muscle leads to impressive results with more than 80% of transfected cells (Dona et al., 2003; Mir et al., 1999), while only a few percent of cells are transfected in tumors (Rols et al., 1998). The mechanisms of gene electrotransfer have been studied on cells *in vitro* and appear to be a multistep process (Golzio et al., 2002): (i) cell membrane permeabilization, (ii) DNA migration towards cell and then insertion in their plasma membrane (Wolf et al., 1994), 2 steps occurring during pulses application, (iii) DNA translocation in the cytoplasm few minutes after pulses delivery, (iv) DNA migration into the cytoplasm towards the nucleus, and finally (v) gene expression occurring a few hours after

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pulses delivery. EGT's electric field parameters have been adapted to allow the efficient membrane permeabilization and the electrophoretic accumulation of plasmid DNA on cells. EGT consists in a low voltage (≤800 V/cm) and long pulse duration (several milliseconds) (Rols et al., 1992) in contrary to ECT conditions (≥1000 V/cm, 100 μs (Rols et al., 1998)).

DNA electrotransfer is therefore a complex phenomenon whose complexity, and the number of limiting steps, are further increased *in vivo*. Cells in culture are not representative of what happens *in vivo* as cells are not structured in tissue. A more complex environment is present in tumor that can prevent the efficient DNA access to cells. The mechanisms of gene transfer in a tridimensional structure as tumor need therefore to be elucidated to propose new strategies to enhance gene expression in tumors. However, study on tumors requires the use of small animal and addressing the process at the cell level *in vivo* is difficult. Therefore we propose to investigate the use of multicellular tumor spheroid (called spheroid or MCTS) as an *ex vivo* model of tumor to investigate DNA electrotransfer in a multicellular structure.

Spheroids present a multicellular tridimensional structural organization (Sutherland, 1988). They display cellular differentiation (linked to nutrient and dioxygen gradient) with quiescent or even dead cells in the core of spheroid and proliferative cells on external layers. These characteristics, all together with extracellular matrix and cell–cell interactions presence, make of the spheroid a good and easy to use model reproducing tumor structure *in vitro*.

Spheroid model was expected to give more information about DNA transfer in tumor than cultured cell in suspension as it is structurally similar to tumor. It has already been used for study and optimization of several gene transfection techniques: cationic polymers (Mellor et al., 2006), lipofection (Gil-Cardeza et al., 2010), photodynamic therapy (Madsen et al., 2006), or cell cycle study (Lobjois et al., 2009). As gene therapy is of growing interest and needs reliable and noninvasive tools, spheroids could help to point out biological, physical and technological barriers to electrotransfection in tumor and allow techniques optimization *ex vivo*. However, this model has only been poorly used in the field of electroporation (Canatella et al., 2004; Mellor et al., 2006). It has already been validated in our group for electrotransfer studies (Wasungu et al., 2009), but the direct comparison with the results obtained in cells in culture and the validation of its capacity to reproduce *in vivo* situation are still missing.

The aim of the present work was to evaluate the use of spheroid as a relevant model for study of electrotransfer process and, by doing so, to give a first answer to the actual gene transfer failure in tumor. To do so, comparison of electro-gene transfer in cells cultured *in vitro* in suspension and cells in spheroids (that mimic *ex vivo* tumor) was done. The key steps of this process were investigated. We addressed different questions concerning cell viability, permeabilization, DNA/membrane interaction, and gene expression. In the interest of optimization, we studied and compared the effect of electric field parameters on cells and spheroids. Electrotransfection was performed at different intensities (from 0 to 800 V/cm) by applying 10 pulses of 5 ms duration at a 1 Hz frequency. Our strategy was to use confocal microscopy as an efficient and non-invasive tool to visualize spheroid behavior (qualitative and spatial analysis). Additionally, flow cytometry was used for quantitative analysis of cells pulsed in suspension or in spheroids (after cell dissociation by enzymatic treatment).

2. Materials and methods

2.1. Cells

The HCT116 cell line was derived from a human colorectal carcinoma. They were selected for their ability to grow plated on Petri dishes and to form spheroids. HCT116 cells were grown in DMEM media (Gibco) containing 4.5 g/l glucose, L-glutamine and pyruvate and supplemented with 10% (v/v) of heat inactivated fetal calf serum, 1% antibiotic mix (100 U/ml penicillin and 100 μg/ml streptomycin) in a 5% CO_2 humidified incubator at 37 °C (Jouan, St. Herblain, France).

2.2. Generation of spheroids

The hanging-drop method has been adapted to produce spheroids of similar diameter (Del Duca et al., 2004). Drops of 20 μl containing 500 cells were suspended on the lid of agar coated 24-well dishes containing 500 μl of culture medium. After 72 h, required for cell aggregation, spheroids were transferred to the agar-coated bottom of the well. Multicellular spheroids were then allowed to grow for 3 days and spheroids used in the experiments sized in average around 500 μm of diameter.

2.3. Plasmid extraction

A 4.7 kb plasmid (pEGFP-C1) containing the gene coding the enhanced Green Fluorescent Protein (eGFP) under control of the CMV promoter was obtained from Clonetech (Palo Alto, CA). It was purified from transfected *Escherichia coli* by using Maxiprep DNA purification system according to Qiagen instructions (Courtaboeuf, France).

2.4. Electropulsation

Electropulsation was achieved by using a CNRS cell electropulsator (Jouan, St. Herblain, France) which delivered square-wave electric pulses. An oscilloscope (Enertec, St. Etienne, France) monitored pulse shape. Stainless steel flat parallel electrodes (1 cm length, 4 mm width) placed on Petri dish were used. Ten pulses lasting 5 ms at a frequency of 1 Hz were applied at different electric field intensity at room temperature, conditions known to induce efficient and transient cell permeabilization (Rols et al., 1992).

2.5. Electropermeabilization

2.5.1. Electropermeabilization of cells in suspension

500,000 cells were resuspended after gentle centrifugation in 100 μL of pulsing buffer (10 mM K_2HPO_4/KH_2PO_4 buffer, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4) containing propidium iodide (PI, 100 μM), placed between electrodes and electric field was applied. After 5 min incubation, penetration of PI was used to monitor permeabilization (Rols et al., 2002). 300 μL of PBS was added to cells in order to determine by flow cytometry (Becton Dickinson FACScan; Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) both the percentage of fluorescent cells (permeabilized cells) and level of fluorescence associated. The excitation wavelength was 488 nm (argon laser) and the fluorescence of intracellular propidium iodide was collected for cell gated with the scatters to exclude debris in FL-2 channel (band pass 585 ± 42 nm). A minimum of 5×10^3 events were acquired in list mode and analyzed with Cellquest software (Becton Dickinson).

2.5.2. Electropermeabilization of spheroids

As for suspension cells, spheroids were placed in 100 μl of pulsing buffer containing PI. After electric pulses application and 5 min incubation at room temperature, spheroids were analyzed with confocal microscopy or flow cytometry. Confocal microscopy studies were realized as follow: spheroids placed in a CoverWell imaging chambers (Sigma Aldrich) in 300 μL PBS (Dubleco's Phosphate Buffer saline) were observed using a Zeiss LSM 510, scanning in *z* axis every 10 μm on 200 μm (excited with a Helium–Neon laser

set at 543 nm wavelength and emitted light collected through a 585 nm long pass filter).

In order to have quantitative data of permeabilization, spheroids were also dissociated 15 min in trypsine–EDTA 1× (Eurobio) and dissociated cells were then analyzed by flow cytometry (Becton Dickinson FACScan; Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA).

2.6. Electrotransfection

2.6.1. Electrotransfection of cells in suspension

As described for electropermeabilization, 500,000 cells were resuspended after gentle centrifugation in 100 μL of pulsing buffer containing 1 μg of plasmid DNA, placed between electrodes and electric field was applied. Cells were cultivated in Petri dish at 37 °C 5% CO_2 in 1.5 mL of culture medium. After 24 h, cell monolayer was washed with PBS to remove all non-adherent cells. Cells were harvested by trypsinization, resuspended in 400 μL PBS and analyzed by flow cytometry to evaluate both the percentage of fluorescent cell (i.e. percentage of eGFP expressing cell, transfected cells) and the mean level of fluorescence associated. The excitation wavelength was 488 nm (argon laser) and the fluorescence of eGFP was collected for cell gated with the scatters to exclude debris in FL-1 channel (bandpass 520 ± 42 nm). A minimum of 5×10^3 events were acquired in list mode and analyzed with Cellquest software (Becton Dickinson).

2.6.2. Electrotransfection of spheroids

Spheroids were taken and added to 100 μl of pulsing buffer containing 10 μg of plasmid DNA already in between electrodes and pulses were applied. Spheroids were cultured in agar coated wells of 24-wells plate with 500 μl of culture medium at 37 °C in a 5% CO_2 incubator for 24 h before confocal analysis (excited with an argon laser set at a 488 nmwavelength and emission collected through a band pass filter from 505 nm to 550 nm) or dissociation and flow cytometer analysis (see Section 2.5).

2.7. DNA/membrane interaction

DNA/membrane interaction was realized for cells in suspension and spheroid as described for electrotransfection but using a fluorescently labeled DNA. Plasmid DNA was stained with the DNA intercalating dye TOTO-1 (Molecular Probe, Eugene, OR): 2.3×10^{-4} M dye was incubated with DNA at a concentration of 1 μg/μL for 60 min on ice. This concentration yields to a ratio of dye to base pair of 1 to 5. Directly after electropulsation, cells and spheroids were observed under confocal microscope (excited with an argon laser set at a 488 nm wavelength and emission collected through a band pass filter from 505 nm to 550 nm).

2.8. Viability

2.8.1. Viability of cells in suspension

It was determined on cells cultured on Petri dish 24 h after electrotransfection. Culture medium was removed and cell monolayer was washed two times with PBS and then 1 mL of 0.1% (m/v) crystal violet solution was added. After 20 min incubation under gentle agitation, crystal violet solution was removed, cell monolayer washed two times with PBS, and then lysed with acetic acid 10% during 10 min. Lyse product was diluted 1/40 before optic density lecture at 595 nm with spectrophotometer (Pharmacia Biotech, Novaspec II). Control DO value is considered as 100% cell viability.

2.8.2. Measure of spheroids growth

As a reflection of cells viability, spheroids growth was quantified over several days. Spheroid growth was followed by taking pictures of spheroids over 9 days with a Leica macrofluo microscope coupled to a coolSNAP HQ camera (Roper scientific, Photometrics). The measure of the projected area of the spheroid on each image was realized and automated using image J software. The relative projected area was expressed as the ratio of the measured area at any given time on the measured area at the beginning of the experiment. This normalized projected area was plotted as a function of time to represent spheroid growth following different treatments.

3. Results

3.1. Comparison between electropermeabilization of cells in suspension and in spheroids

Membrane destabilization induced by electric field leads to small molecules transfer into cells. Using this ability, the entry of the fluorescent probe propidium iodide (PI) was used to monitor permeabilization (Rols et al., 2002). The permeabilization of cells in suspension and cells in spheroids was studied as presented in Fig. 1. In Fig. 1A, permeabilization of cells pulsed in suspension is reported. Two parameters are of interest: percentage of fluorescent cells (Fig. 1A, left panel), that represents the fraction of cells that have been permeabilized, and fluorescence level inside the cells (Fig. 1A, right panel), that is directly proportional to the amount of molecule incorporated into the cells. Results show that membrane permeabilization depends on electric field intensity. Cells are permeabilized above a threshold value, situated between 300 and 400 V/cm, 400 V/cm conditions giving significant number of cell permeabilized in comparison with control (34.49% ± 9.741 SEM). Below that threshold, the percentage of fluorescent cells is similar to the one of the control, i.e. in absence of electric field where less than 5% of cells are permeable representing dead cells with loss of membrane integrity. In terms of fluorescence intensity, substantial accumulation of PI inside cells is detected between 400 V/cm and 500 V/cm (1187 a.u. ± 232.1 SEM, significant in comparison of 400 V/cm condition, Fig. 1A right panel). From 300 to 800 V/cm, the number of permeable cells increases to reach more than 80% above 500 V/cm (82.15% ± 5.085 SEM for 600 V/cm significant in comparison of 400 V/cm condition). The amount of PI inside cells increases from 300 V/cm to 700 V/cm. Under those experimental conditions, up to 80% of cells should be potentially transfectable.

Electropermeabilization of cells present in spheroid was then studied by using both confocal microscopy to have a global qualitative view, and flow cytometry to quantify the cell response at the single cell level after dissociation of the cells from the spheroids. Images obtained by confocal microscopy are presented in Fig. 1B. 3D projections of confocal acquisitions for different spheroids subjected to increasing electric field intensity are shown. This allowed us to visualize the spatial distribution of permeabilized cells in the spheroids. The number of permeabilized cell increases with the electric field intensity homogeneously at the spheroid surface. Permeabilization is detected for field intensities as low as 200 V/cm. At 500 V/cm, all the cells on the spheroid surface are fluorescent. In Fig. 1C, optical slices in *z* axis show that fluorescence signal in the spheroid core decrease. Inner cells are not detected from 40 μm depth and only external cells layers are visible. It was therefore impossible to assess the permeabilization state of the cells deep in the core of the spheroid, as this decrease can be due to the absence of permeabilization or to technical limitation. To address this issue, we next performed spheroid enzymatic dissociation that enabled us to use flow cytometry to quantify permeabilization of all cells of the spheroid. As shown in Fig. 1D, a maximum of 30.31% ± 4.992 SEM of cells are permeabilized in the spheroid at 400 V/cm (29.59% ± 3.809 SEM at 300 V/cm, both conditions being significant in comparison with the control). Above this electric

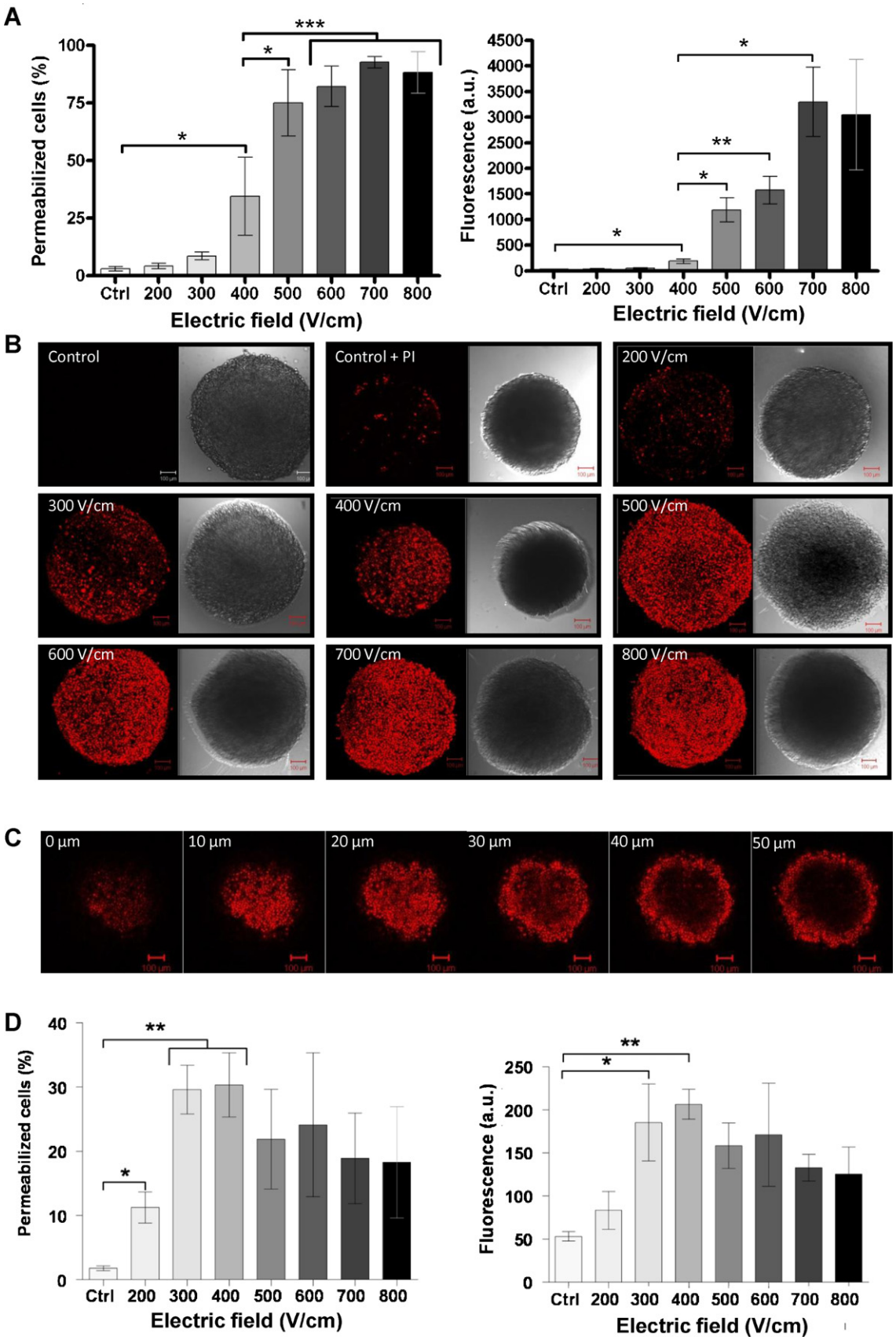

Fig. 1. Electropermeabilization of cells in suspension and in spheroids. (A) HCT116 cell permeabilization assessed with propidium iodide uptake (triplicate, mean ± SEM, ANOVA test) and fluorescence analysis for different electric field intensity (triplicate, mean ± SEM, Unpaired Student's *t*-test). (B) 3D projection of permeabilized spheroid confocal acquisitions (propidium iodide uptake) and phase contrast corresponding images. This experiment was performed 3 times and one characteristic picture is presented here. For all images scale bar represents 100 µm. (C) Optical slices of confocal acquisition for 800 V/cm and fluorescence signal decrease from 40 µm depth. (D) Permeabilized cell percentage in dissociated spheroids assessed with propidium iodide uptake and matching fluorescence intensity depending on electric field intensity (triplicate, mean ± SEM, Unpaired Student's *t*-test). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

field intensity, this percentage decreases to 21.87% ± 7.774 SEM at 500 V/cm for example, not significant compared to 400 V/cm. The associated fluorescence intensity curve follows the same profile reaching a maximum for 400 V/cm (206.5 a.u. ± 17.88 SEM significant compared to control).

3.2. Observation of DNA/membrane interaction on single cells in suspension and cells in spheroids

DNA/membrane interaction is a key step in DNA electrotransfer. Submitted to electrophoretic forces, plasmid DNA migrates towards the cells and forms aggregates in the electropermeabilized region of the membrane (Golzio et al., 2002). These aggregates remain visible several minutes after electric field application. Images of these particular structures can be obtained with a confocal microscope using TOTO-1 fluorescently labeled plasmid.

For single cell in suspension, results are coherent with earlier published studies showing DNA/membrane interaction spots on the side of the cell facing the cathode (Fig. 2A). On spheroids, our studies of DNA/membrane interaction shows for the first time the same process, i.e. interaction of DNA with cells present in a tissue model as shown in Fig. 2B. Interestingly, the interaction of DNA on spheroid's cells happens on one hemisphere. As for single cells, it corresponds to the hemisphere facing the cathode (Fig. 2B-1). Moreover, aggregates are visible as for singles cells (Fig. 2B-2). Acquisitions in *z*-axis revealed that this interaction takes place only on the cells present on the external cell layer of the spheroid (Fig. 2C). Indeed, each optical slice presents a different fluorescence profile. In comparison with permeabilization picture (Fig. 1C), where at 15 μm depth cells can be detected, DNA is detected only on one cell monolayer surrounding spheroid before 15 μm depth. This demonstrates that DNA does not interact with inner cells.

3.3. Comparison of electrotransfection between single cells in suspension and spheroids

Electrotransfection is carried out with a plasmid coding the enhanced green fluorescent protein (eGFP). Transfection rate is evaluated 24 h after electrotransfection by visualizing eGFP expression with confocal microscopy and quantifying it with flow cytometry. 10 times more plasmid is used for spheroid transfection than for cells in suspension as shown in Wasungu et al. (2009). This higher amount of DNA was expected to allow some plasmid DNA to cross cellular barrier (first layers of cell) and reach inner cells.

Flow cytometry analysis performed in cells pulsed in suspension is presented in Fig. 3A. As for cell permeabilization, transfection is observed above 300 V/cm intensity, reinforcing the idea that permeabilization is a necessary condition for plasmid uptake (Wolf et al., 1994). Percentage of cells expressing eGFP is increasing by increasing the electric field strength and reach 23.75% ± 2.450 SEM at 500 V/cm (significant compared to 400 V/cm). Transfection's efficacy, assessed by fluorescence intensity, is maximal at 500–600 V/cm (17.07 a.u. ± 3.150 SEM at 500 V/cm, significant compared to control but not to 600 V/cm). Above that value, both transfection rate and efficacy decrease because of cell damage due to a too high field and/or DNA cytotoxicity.

In spheroids, eGFP expression is only detected for electric field values close to 400 and 500 V/cm (Fig. 3B). These values allow only a few cells to express eGFP. eGFP expressing cells are localized on one spheroid side, according to the observation of DNA/membrane interaction. No significant results have been observed with flow cytometry analysis as very few cells are transfected (data not shown).

3.4. Viability of cells in suspension and spheroids growth

The viability of cells in suspension was measured 24 h after pulse application. As shown in Fig. 4A, cell viability starts to be affected above 400 V/cm: 64.66% ± 8.530 SEM of cells are still viable at 500 V/cm. Above this intensity, the cell viability is much more affected (only 24.77% ± 5.061 SEM of cells being viable at 600 V/cm and 3.813% ± 0.5138 SEM at 800 V/cm).

Spheroids viability was evaluated by their ability to grow over a period of 9 days. Only three characteristic conditions (control, 500 V/cm and 800 V/cm) are presented in Fig. 4B. Control conditions show the normal growth behavior of the spheroids: spheroids diameter increases by a factor 5 during this period. When submitted to 500 V/cm intensity electric field pulses, the spheroid growth stops during the first 48 h and then goes back to a normal growth rate. This may reflect some cell mortality on the external proliferative cell layers. With the more drastic condition of 800 V/cm, there is a decrease in spheroid size, indicating the loss of a large number of cells most probably on the external layer. Spheroids reach back their normal growth rate after 6 days. Those results show that viability is affected by electrotransfection, especially at higher electric field intensity and gives a first explanation to the poor transfection efficiency.

4. Discussion

Electrotransfection is a complex process involving at least 5 consecutivessteps: membrane permeabilization, DNA electrophoresis and interaction with the destabilized cell membrane, DNA entry in cell, nuclear envelope crossing and expression (Escoffre et al., 2009). Difference of efficacy between *in vitro* and *in vivo* studies points out that other limiting step could be present *in vivo* and that technique's optimization is still needed. The spheroid model was expected to give more information about DNA transfer in tumor than cultured cell in suspension as it is structurally similar to tumor. In this paper, we have studied and compared DNA transfer in cells in suspension and in spheroid in order to address the use of spheroidas a relevant model in electrotransfection studies.

Behavior of cells in response to electric field is totally different for cells in suspension and spheroids. For isolated cells, 500 V/cm pulses can lead to 74.96% ± 8.269 SEM of permeabilization, 23.75% ± 2.450 SEM of transfection while maintaining cell viability up to 64.66 ± 8.530 SEM. Therefore this electric field value seems to be the optimum electric field intensity for HCT116 cells in suspension, leading to a rather good transfection level associated to high permeabilization and viability. For spheroids, all cells cannot be permeabilized by electric field (at least in conditions allowing propidium iodide uptake). Permeabilization upper limit around 30% indicates that, whatever the electrical field conditions, only a fraction of cells can be efficiently permeabilized. Combining these data with confocal observations lead to think that only the external cells layers can be permeabilized and that these cells represent one third of the spheroid. This can be due to electric field property inside the spheroid structure, effect on transmembrane potential being modified into such complex structure (Pavlin et al., 2002). Moreover, cells shape in spheroid is strongly affected by cell–cell contact and far from ovoid shape of cells in suspension. That is in agreement with the fact that cell shape and orientation affect electric field effect (Valic et al., 2003). Another possible explanation is that PI can have a reduce accessibility to cells present in the core of the spheroid. A third reason for this limited permeabilization is related to the cellular state. Due to nutrient and dioxygen gradient, cells in spheroid core may be apoptotic or necrotic (Sutherland, 1988) and consequently not able to accumulate PI intracellularly due to their leaky membrane. To conclude, the 3D and multicellular structure

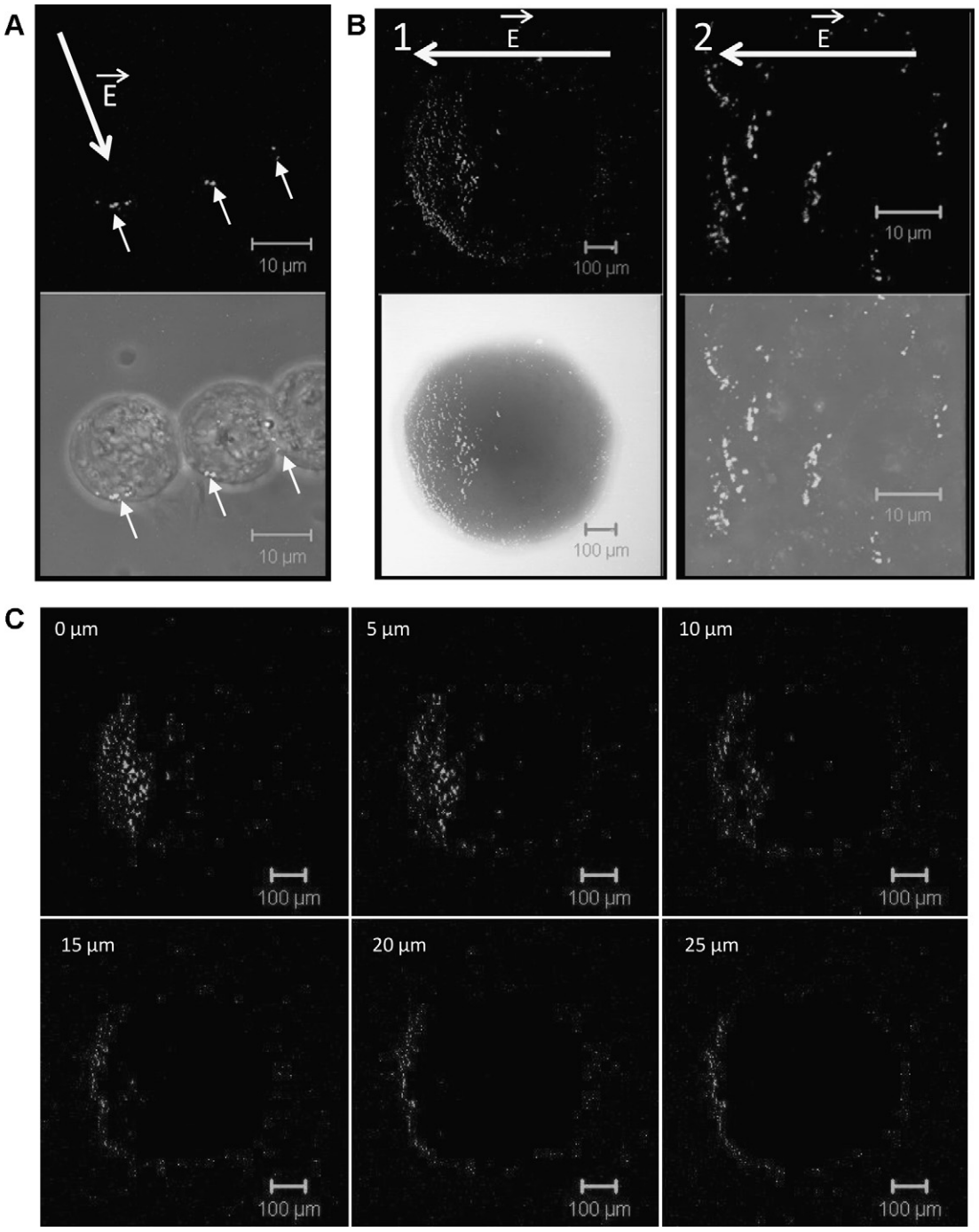

Fig. 2. DNA/membrane interaction on single cells in suspension and cells in spheroids. Cells in suspension and spheroids were electropulsated (10 pulses of 5 ms, 1 Hz at 500 V/cm) in the presence of fluorescently labeled plasmid DNA. (A) 3D projection of confocal acquisitions and phase contrast of cells in suspension. Small arrows point to DNA aggregates and large arrow indicates electric field direction. The scale bar represents 10 μm. (B) 3D projection of confocal acquisitions and phase contrast of electropulsated spheroid. This experiment was performed 3 times and one characteristic picture is presented here. Large arrow indicates electric field direction. 1: entire spheroid, scale bar is 100 μm, 2: zoom in, scale bar is 10m. (C) Confocal slice of an electropulsated spheroid between the surface of the spheroid and 25 μm depth with 5 μm step, showing interaction only on external layer. The scale bar represents 100 μm.

of spheroid that mimics tumor organization, with the presence of extracellular matrix, can be responsible for the limited permeabilization. Electrical field conditions have therefore to be optimized. Since increasing the voltage leads to a decrease in cell viability, another strategy consists in applying shorter pulses (several hundred of microseconds instead of 5 ms) in order to apply higher electric field values. This is actually what is already done in electrochemo-therapy protocols using shorter pulse and higher voltage (Marty et al., 2006). Under these conditions we have observed that all cells can be permeabilized even the ones present inside spheroid (personal communication). EGT conditions are therefore less efficient on 3D structure even when optimized on cultured cells.

Another key point of this paper is the DNA/membrane interaction that has been observed for the first time on a tissue-like structure. Observation of unique cells in spheroid was possible and highly improved the analysis of the mechanism. Pictures of fluorescent plasmid DNA show that it cannot cross all cell layers to reach the core of the spheroid even at high concentration (10 times

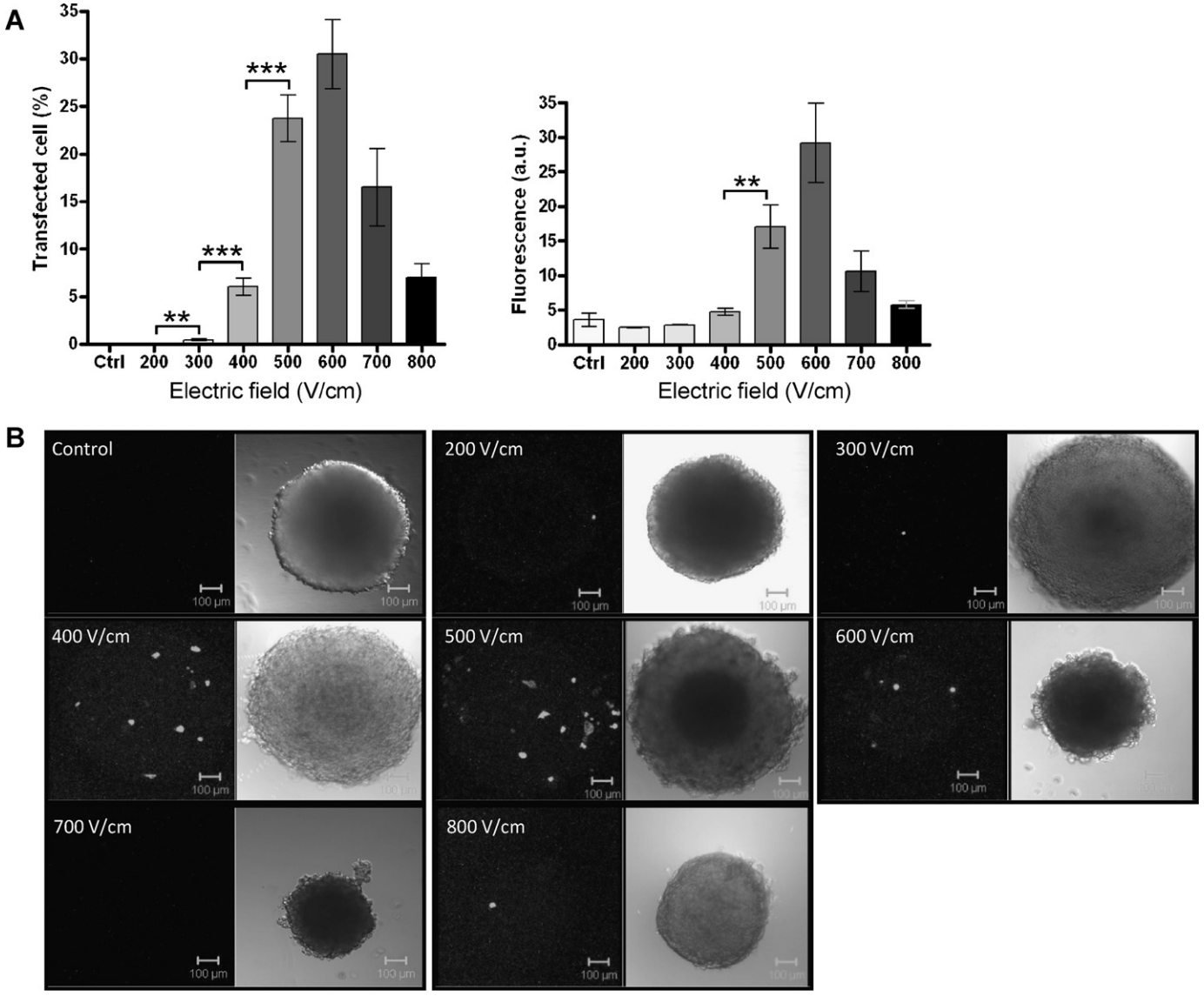

Fig. 3. Comparison of electrotransfection between single cells in suspension and spheroids. (A) Transfection rate of cell in suspension assessed with eGFP expression and matching fluorescence intensity depending on electric field intensity (triplicate, mean ± SEM, ANOVA test). (B) 3D projection of confocal acquisitions and phase contrast of spheroid electrotransfected with DNA coding eGFP at different electric field intensity (as indicated). This experiment was performed 3 times and one characteristic picture is presented here. The scale bar represents 100 µm. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

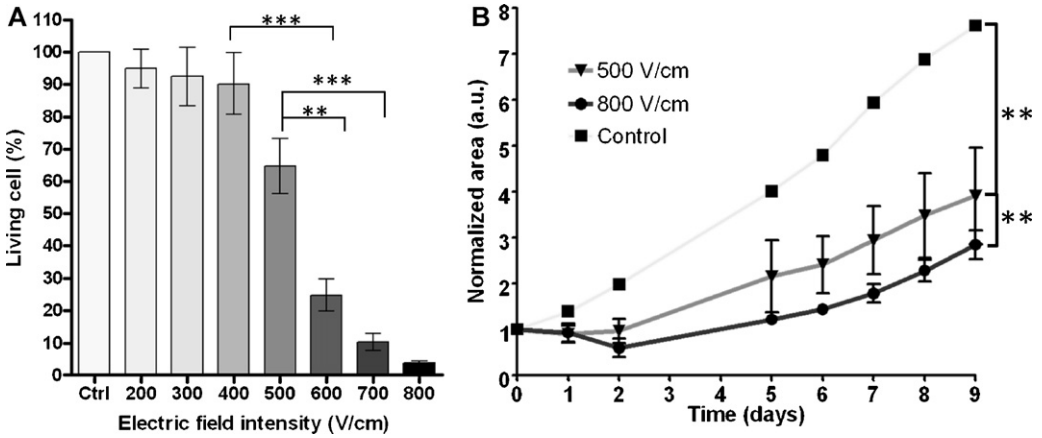

Fig. 4. Electric field effect on cell viability. (A) Viability of cell in suspension 24 h after electrotransfection depending on electric field intensity (triplicate, mean ± SEM, ANOVA test). (B) Spheroid growth curves with DNA during electric field application. For easier reading, only control, 500 and 800 V/cm are presented here (triplicate, mean ± SEM, Paired Student's *t*-test) $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

more than the one used for cells in suspension). This is probably the first cause of poor transfection results in tumor. While 80% of transfection rate is obtained in muscle tissue, in murine tumor only 5% of cells are expressing transgene. Interaction between tumoral cells must be specific and create a so dense environment that DNA cannot pass through them (Smrekar et al., 2003; Zaharoff et al., 2002). The same behavior seems to happen in spheroid, showing that DNA/interaction results can be extrapolated to tumor and that the spheroid model is relevant to mimic tumor. This limited number of cells in interaction with DNA gives another explanation for the poor transfection. Indeed less than 1% of cells are transfected. This last result encourages reconsidering DNA amount used. Indeed, the higher DNA amount (100 μg/ml), initially chosen to allow some plasmid DNA to cross cellular barrier (first layers of cell) and reach inner cells, do not have any of the expected effect. Therefore, attention should be paid on DNA amount for optimization of transfer conditions. Other ways to improve plasmid DNA biodistribution inside the spheroids should be explored such as electrophoresis, to force and direct DNA migration, or the addition of enzymes to disrupt junctions between cells.

Finally, viability results can also explain the poor expression of transgene. As Fig. 4 shows, growth is strongly affected by electric pulse, meaning that those electric fields are killing the entire proliferative cell layer. Spheroid structure presents the proliferative cells on the last external layers. Therefore proliferative cells are the ones potentially transfectable (permeabilized and in contact with DNA) but also the ones affected by electropermeabilization. Around 30% of cells are permeabilized and half of them get contact with DNA, we should consequently expect to have near 15% of transfected cells. However, due to cell death, only a few cells are viable and permeabilized, leading to 1% of expressing eGFP cells.

Spheroid is a safe and easy to use model as it is an *ex vivo* (and *in vitro*) one. This model allows us to visualize directly electric field effect on a complex tridimensional structure close to tissue and preventing small animal use. As in tumor, nutrient and oxygen gradient are presents, creating different physiological cell state (dead, quiescent, proliferative cells). Extracellular matrix and cell interconnection are present as well (Santini et al., 2000). These characteristics are specific to multicellular tumor spheroid (and tumor) and make of it an interesting and useful *in vitro* model more close to tumor tissue than cell monolayer (Friedrich et al., 2007). MCTS could offers possibility to mix different type of cells (Nakamura et al., 1999) and so to get closer to tumor environment. The absence of blood vessel is one of its the major drawbacks, positioning proliferative cells on the external layers (Sutherland, 1988). Eventually, although spheroid is a simple representation of tumor, it is cheaper and more ethic than mouse and allow single cell study, quantification and direct observation of cell behavior. Despite its drawbacks, results obtained with spheroid are nicely reproducing *in vivo* situation. We showed here that less than 1% of spheroid cell can express transgene and that this may be caused both by electric field (low voltage, long pulse duration parameters) and multicellular structure. As previously described (Canatella et al., 2004), electric field is locally reduced in spheroid because of cell size and prevent permeabilization of cells. Furthermore, the cells themselves, their junctions and the secreted extracellular matrix will hinder DNA electrophoresis. Even if this process is inverted in spheroid as DNA is all around the spheroid and not injected inside (like in tumor protocol), the physical cell barrier should be the same in both case, and *in vivo* transfected cells appear in external layers too (Mesojednik et al., 2007). As shown in other studies (Goodman et al., 2007), collagenase treatments help nanoparticles enter deeper in the structure, pointing out the importance that has the extracellular matrix in passive and active passages through cells layers. Nevertheless, the optimal electric field condition of 500 V/cm (using 10 pulses of 5 ms, 1 Hz) is the same for cells in suspension and spheroid, this value giving the best transfection rate in both models.

5. Conclusion

Reproducing *in vivo* situation, spheroids clearly mimic *in vivo* tumor results, opening up possibility of optimization *ex vivo* before *in vivo* tests on small animals. A lot of tumoral cell type can be cultured in spheroid (Freyer and Sutherland, 1980; Grill et al., 2002; Kelm et al., 2003; Kumar et al., 2008) and so characterization and optimization could be finely tuned for each tumor type, allowing the emergence of specific conditions depending on the cell type.

Spheroid will enhance our understanding of the underlying process of DNA electrotransfer, give explanations to *in vivo* limitation of the technique and help in the emergence of new protocols.

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