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3D Spheroids' Sensitivity to Electric Field Pulses Depends on Their Size

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Abstract Dramatic differences of cells behavior exist between cells cultured under classical 2D monolayers and 3D models, the latter being closer to in vivo responses. Thus, many 3D cell culture models have been developed. Among them, multicellular tumor spheroid appears as a nice and easy-to-handle 3D model based on cell adhesion properties. It is composed of one or several cell types and is widely used to address carcinogenesis, or drugs screening. A few and recent publications report the use of spheroids to investigate electropermeabilization process. We studied the response of spheroids to electrical field pulses (EP) in terms of their age, diameter or formation technique. We found that small human HCT-116 colorectal spheroids are more sensitive to electric field pulses than larger ones. Indeed, the growth of spheroids with a diameter of 300 µm decreased by a factor 2 over 4 days when submitted to EP (8 pulses, lasting 100 µs at a 1,300 V/cm field intensity). Under those electrical conditions, 650 µm spheroids were not affected. These data were the same whatever the formation method (i.e. hanging drop and nonadherent techniques). These observations point out the fact that characteristics of 3D cell models have to be taken into account to avoid biased conclusions of experimental data.

Keywords 3D · Electrochemotherapy · Electropermeabilization · Growth · Size · Spheroid

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Accumulating evidences underline that 3D cell models are superior to classical 2D cell culture to mimic and predict in vivo situations (Ghajar and Bissell 2010; Nyga et al. 2011; Pampaloni et al. 2007). Indeed, some studies found differences in drug sensitivity (Chitcholtan et al. 2012), proliferation (dit Faute et al. 2002), extracellular matrix production (Kelm et al. 2010) and gene expression profile (Ghosh et al. 2005) between 2D and 3D in vitro cell cultures.

In that context, Sutherland (1988) developed in the 1980s a 3D cellular model devoid of any exogenous material named spheroid. It is based on cell aggregation properties. Spheroid formation can be initiated in a microgravity environment within a bioreactor (Marrero et al. 2009), using spinner flask or gyratory shaker cultures (Canatella et al. 2004), with the hanging drop technique (Kelm et al. 2003), or by seeding cells in nonadherent 96-well plates (Wenger et al. 2005) or in 96-well plates coated with agarose (Ma et al. 2012) or poly-HEMA (Ivascu and Kubbies 2006). In spheroid, cells develop intercellular junctions and secrete extracellular matrix components such as collagens, laminin, fibronectin and glycosaminoglycans (Kunz-Schughart et al. 2006; Nederman et al. 1984; Stevens et al. 2009). Spheroid applications are widely developed to assess invasion (Hattermann et al. 2011), angiogenesis (Correa de Sampaio et al. 2012), chemotherapeutic agents screening (Vinci et al. 2012), novel drug delivery systems (Kim et al. 2010), cell–cell interactions (Santini et al. 2000), and to investigate therapeutic potential of treatment (Upreti et al. 2011).

Among all the delivery methods, a physical approach named electropermeabilization, or electroporation, is now accepted in clinics (Kee et al. 2011; Matthiessen et al. 2012; Mir et al. 1998; Sersa et al. 2011). This technique showing that cell membrane can be efficiently transiently permeabilized under application of electric pulses has been introduced by Neumann in the 1970s (Neumann and Rosenheck 1972).

Electroporation allows the penetration of molecules (cytotoxic drugs, DNA) into cells and tissues. Activity of anticancer drugs can be potentiated, a process called electrochemotherapy (ECT). Today ECT is safely and currently used in human clinics for the treatment of melanoma and head and neck cancer (Kee et al. 2011; Mir et al. 1998) as well as in veterinary medicine (Pavlin et al. 2012; Tamzali et al. 2012). Hundreds of in vitro studies on electropermeabilization process performed on cells in culture allowed to optimize the electric parameters and thus to apply this technique in clinics. However, even though they are useful, these studies do not address the complexity of in vivo tissues. This is why we and few others proposed to employ spheroids to in vitro study electrotransfer of (macro)molecules in a 3D context. Thus, Canatella et al. (2004) used human prostate carcinoma spheroids grown in spinner flasks to assess molecular uptake in a densely and heterogeneously packed cells environment. Mellor et al. (2006) built human colon carcinoma spheroids grown in agarose-coated wells to in vitro optimize nonviral gene delivery. Our group used human colon carcinoma spheroids grown by the hanging drop technique to in vitro mimic electrochemotherapy process (Gibot et al. 2013) and to study electrogene transfer mechanisms (Chopinet et al. 2012; Wasungu et al. 2009). More recently, Heller's group developed large human keratinocytes spheroids in a microgravity environment to predict in vivo plasmid transfection by electroporation (Marrero and Heller 2012).

All these studies were led with spheroids composed of diverse cell types, with different diameters, and formed by distinct methods. The aim of our present study was to evaluate the effect of spheroid characteristics on their response to electric pulses. We focused on 3 parameters: age, diameter and formation technique. For this purpose, we used standard electrical parameters defined by the ESOPE (European standard operating procedures on electrochemotherapy) study, a multi-institutional human clinical study on small skin tumors treatment by ECT (Marty et al. 2006).

Materials and Methods

Cell Culture

The HCT-116 cell line, a human colorectal carcinoma, was purchased from ATCC (CCL-247). These cells were chosen for their ability to form multicellular tumor spheroids (MCTS). HCT-116 cells were grown in Dulbecco modified Eagle medium (Gibco-Invitrogen, Carlsbad, USA) containing 4.5 g/l glucose, L-glutamine and pyruvate, supplemented with 10 % (v/v) of heat inactivated fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2.

Generation of Spheroids by the Hanging Drop Technique

The hanging drop technique for spheroid generation was adapted from Kelm et al. (2003) to produce spheroids with an homogeneous diameter. Briefly, 20 µl drops containing 500 cells or 5,000 cells were placed on the lid of agar coated 48-well dishes containing 250 µl of culture media. After inversion, drops hanging from the lid by surface tension allowed cells sedimentation. After 72 h, time required for cell aggregation, spheroids were transferred to the agar-coated bottom of the well containing 250 µl of fresh culture medium. Multicellular spheroids were then allowed to grow for 2 or 7 more days and the spheroids used for experiments were 5 or 10 days old.

Generation of Spheroids by the Nonadherent Technique

We used ultra-low attachment 96-well plates from Corning (Fisher Scientific, Illkirch, France). Briefly, 500, 2,500 or 5,000 cells in suspension were seeded in 150 µl of medium in each well. Plate was centrifuged 5 min at 4 °C at $300\times g$ in order to accelerate cell sedimentation. Spheroids were cultivated for 5 days at 37 °C in a humidified atmosphere containing 5 % CO_2.

Electropermeabilization of Spheroids

Spheroids were placed between two stainless steel flat parallel electrodes (1 cm length, 0.4 cm width) 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). Electropulsation was achieved by using an ELECTRO cell S20 generator (βTech, Toulouse, France) which delivered square-wave electric pulses. An oscilloscope (Enertec, St. Etienne, France) was used to monitor pulse shape. The electrical conditions were the following: 8 pulses lasting 100 µs at a frequency of 1 Hz were applied at a 1,300 V/cm electric field intensity at room temperature. These conditions are the ones used under ECT ESOPE protocols (Kee et al. 2011).

MCTS Growth Curve

MCTS growth was followed by taking photographs of the spheroids before and over five days after electric field application with a Leica macrofluo microscope coupled to a coolSNAP HQ camera (Roper Scientific, Germany). The size of the projected area of the MCTS on each image was obtained using image J software (NIH, Bethesda, USA). The normalized area was expressed as the ratio of the measured area at any given time on the measured area before the beginning of the experiment.

Statistical Analysis

For every set of experiments, 5–6 biological replicates have been produced, treated and analyzed. Differences between values were assessed by one way ANOVA using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, USA). All data were expressed as mean ± SEM, and overall statistical significance was set at $p < 0.05$.

Graphic Program

Graphic program used to create the artwork was GraphPad Prism.

Results

In order to evaluate if the characteristics of 3D cell models may influence their responses, we followed the growth of spheroids submitted or not to electric field pulses. For this purpose, we visualized the same spheroids over the time by using phase contrast microscopy as an appropriate tool to directly check their morphology and to measure their surfaces. Spheroids composed of human colon carcinoma cells HCT-116 were submitted to electric fields pulses optimized for in vivo ECT. The EP parameters were as follows: 8 pulses of 100 µs, 1,300 V/cm applied at a 1 Hz frequency (Kee et al. 2011). Since spheroid growth capacity decreased when their diameter increased, we worked with proliferating spheroids, i.e. spheroids <700 µm in diameter.

Spheroid Response to Electric Field Pulses Depends on Their Characteristics

Spheroids formed with 500 cells by the hanging drop technique were grown for two different times: 5 and 10 total days (Fig. 1a). At these time points, diameters were respectively close to 300 ± 4 µm and 660 ± 4 µm (Fig. 1b). In order to determine the effects of electric pulses on spheroids, we quantified their growth over 4 days. Thus, spheroids were either submitted ("EP") or not ("Control") to electric pulses applied in ECT protocols. For the day 5 experiment (Fig. 1c), untreated spheroids (Control) displayed a normal growth where their projected surface increased linearly by 160 % over 4 days. When submitted to electric field pulses (EP) their growth only increased by 25 %, showing that electric field has a drastic effect on spheroid growth capacity. For experiments performed at day 10 (Fig. 1d), both control and electrically treated spheroid surface increased linearly by 60 %. In that case, electrical field pulses had no effect on spheroid growth capacity. In order to discriminate if the differential response to electric field came from the age/maturation or the diameter/size of the spheroid, we seeded new spheroids with 5,000 cells. Their diameters at day 5 were about 530 ± 10 µm. In that case, control spheroid surface increased linearly by 60 % while electrically treated spheroid only increased by 20 % (Fig. 2). These results show that the smaller the spheroids are, the more EP affect their growth capacity. Moreover, the older the spheroids are, the less sensitive they appear.

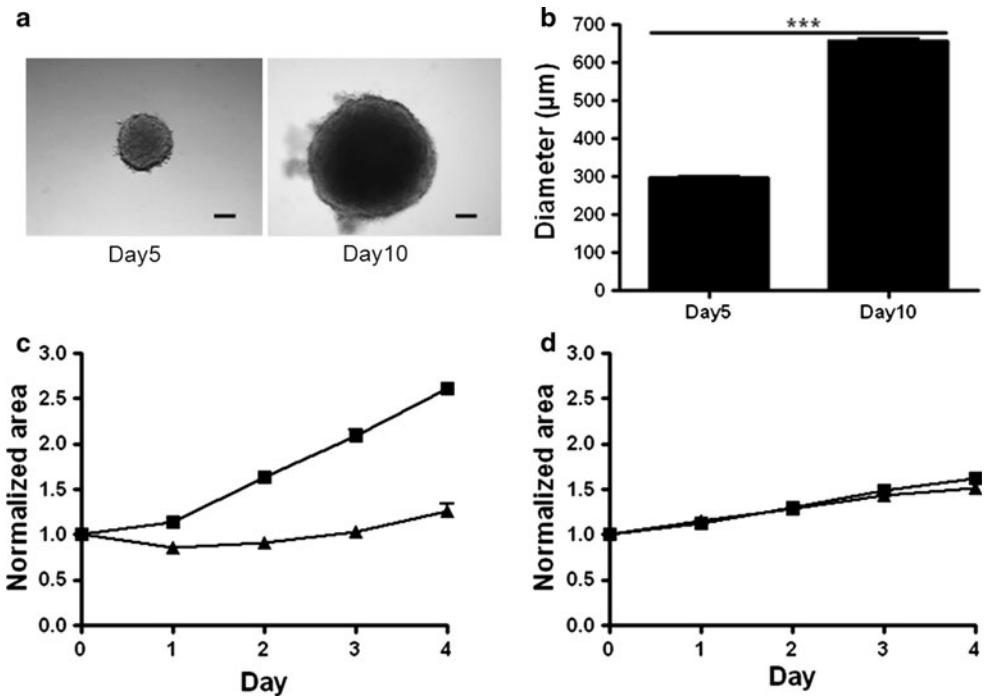

Fig. 1 Spheroid seeded with 500 cells by the hanging drop technic. **a** Macroscopic view of spheroids at 5 and 10 days of culture. **b** Spheroid diameters. **c** Spheroids growth curve when pulsed at day 5. **d** Spheroid growth curve when pulsed at day 10. *** $p < 0.0001$. *Scale bar* 100 µm. *Squares* represent control condition; *triangles* represent electropulsated condition ($n = 5$)

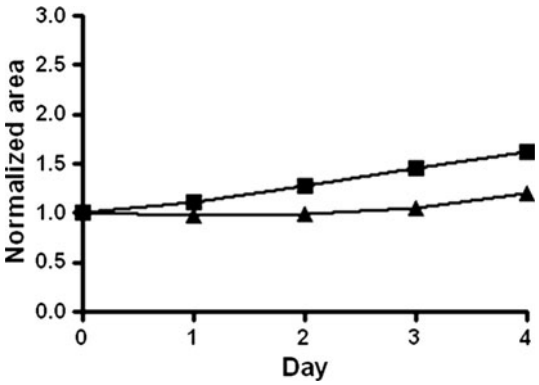

Fig. 2 Growth curve of spheroid seeded with 5,000 cells by the hanging drop technic when pulsed at day 5. *Squares* represent control condition; *triangles* represent electropulsated condition ($n = 5$)

Spheroid Size Affects Sensitivity to Electric Field Pulses

In order to determine if the sensitivity of spheroids to EP depended on its size whatever the method used to obtain them, we formed spheroids of different diameters by the nonadherent technique. Spheroids seeded with 500 cells, 2,500 cells and 5,000 cells had respectively a diameter close to 290 ± 11, 410 ± 11 and 460 ± 11 µm (Fig. 3a). Growth was followed over 5 days after electrical treatment. For the smallest spheroid seeded with 500 cells, surface linearly increased by 250 % for the control and only by 80 % for those electropermeabilized (Fig. 3b). Intermediate spheroids seeded with 2,500 cells saw their surface linearly increased by 140 % for the control and only 30 % for those submitted to electric field. Finally, for the largest spheroids seeded with 5,000 cells, surface linearly increased by 90 % for the control and 30 % for the treated ones. Thus, as previously observed with spheroid formed by the hanging drop technique, the smaller the spheroid's diameter, the more EP affected its growth.

Discussion

In this study we showed that under standard electrical parameters applied in ECT protocols, the growth of small spheroids having a diameter under 300 µm was dramatically reduced compared to the largest ones with diameters around 500–600 µm. This observation was true whatever the formation technique used (i.e. hanging drop and non-adherent technique). These results can be explained by the fact that the growing rate of small spheroids is higher than the growing rate of larger ones. It is indeed known the tumor spheroid 3D cultures display chemical gradients (e.g. oxygen, nutrients, and catabolites) (Sutherland et al. 1986). For diameters larger than 500–600 µm, spheroids develop central necrosis core while proliferating cells are localized on external layers (Sutherland and Durand 1984).

In 2004, Canatella et al. underlined in a 3D spheroid model a local perturbation of the electric field repartition due to heterogeneous high-density multicellular environment electrical properties. Indeed, the influence of cell density on cell membrane electropermeabilization was numerically and experimentally assessed in simple tissue model and showed that an increased cell density induced a decrease in the amplitude of the induced transmembrane voltage (Pavlin et al. 2002; Pucihar et al. 2007).

Furthermore, cells within spheroids could be as much as 30 % smaller than peripheral cells, probably due to nutrients

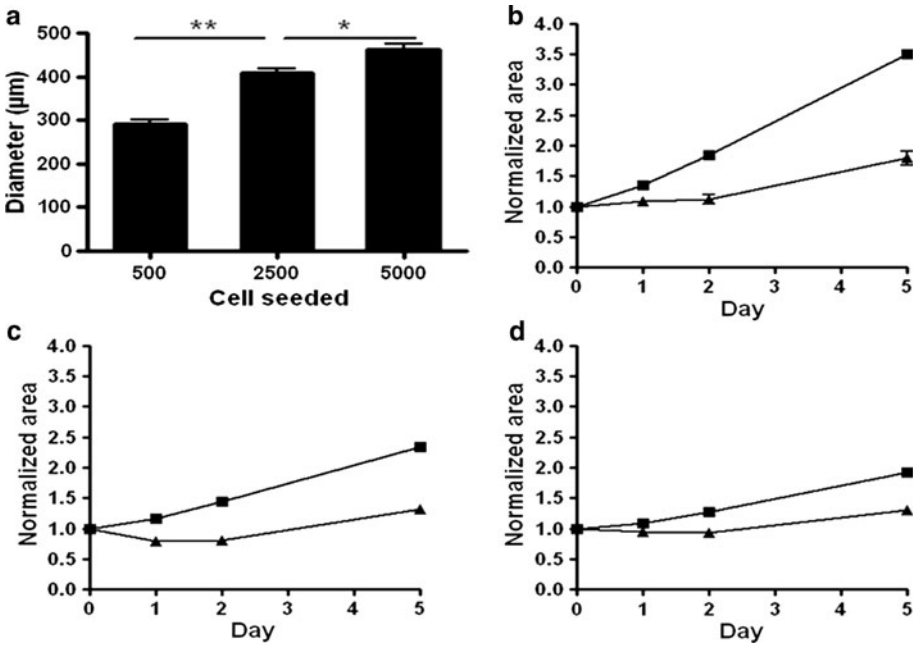

Fig. 3 Spheroid seeded with 500, 2,500 or 5,000 cells by the nonadherent technic pulsed at day 5. **a** Spheroid diameters. **b** Spheroid seeded with 500 cells growth curve. **c** Spheroid seeded with 2500 cells growth curve. **d** Spheroid seeded with 5,000 cells growth curve. ** $p < 0.001$, * $p < 0.05$. *Squares* represent control condition; *triangles* represent electropulsated condition ($n = 6$)

and oxygen diffusion distance from spheroid surface (Mueller-Klieser and Sutherland 1984; Sutherland 1988). Canatella et al. (2004) showed with spheroids ranging from 100 to 400 μm in diameter that interior cell radii were up to 19 % smaller than peripheral cells. Because at a constant electric field intensity the induced transmembrane voltage decreases with cell radius (Escoffre and Rols 2012), smaller cells within the spheroid interior underwent weaker electropermeabilization than larger peripheral cells.

Thus, in our conditions, the fact that large spheroids were less sensitive to EP could be due to the inhomogeneity in electrical field repartition because of position-dependent variation in cell size and heterogeneous electrical properties in spheroid with necrotic center. Some others aspects have also to be taken into account. One have to confirm that in a 3D context proliferating cells are more sensitive to EP than quiescent cells. Therefore, since spheroids' characteristics changed with maturation/aging, it will be interesting to further assess the role of extracellular matrix secretion and organization, as well as intercellular junction and communication, in their sensitivity to electric pulses.

As a general conclusion, these data point out the fact that the characteristics of 3D cell models, in terms of size and proliferation, have to be taken into account to avoid biased interpretations of experimental data.

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