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Antitumor drug delivery in multicellular spheroids by electropermeabilization ☆

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ABSTRACT

Electrochemotherapy (ECT) is a physical technique that allows cytotoxic molecules to be efficiently released in tumor cells by inducing transient cell plasma membrane permeabilization. The main antitumoral drugs used in ECT are nonpermeant bleomycin and low permeant cisplatin. The method is nowadays applied in clinics as a palliative treatment. In order to improve it, we took advantage of a human 3D multicellular tumor spheroid as a model of tumor to visually and molecularly assess the effect of ECT. We used bleomycin and cisplatin to confirm its relevance and doxorubicin to show its potential to screen new antitumor drug candidates for ECT. Confocal microscopy was used to visualize the topological distribution of permeabilized cells in 3D spheroids subjected to electric pulses. Our results revealed that all cells were efficiently permeabilized, whatever their localization in the spheroid, even those in the core. The combination of antitumor drugs and electric pulses (ECT) led to changes in spheroid macroscopic morphology and cell cohesion, to tumor spheroid growth arrest and finally to its complete apoptosis-mediated dislocation, mimicking previously observed in vivo situations. Taken together, these results indicate that the spheroid model is relevant for the study and optimization of electromediated drug delivery protocols.

1. Introduction

The efficient and safe delivery of therapeutic molecules is still a challenge in medicine and especially in cancer therapy where cytotoxic drugs have to reach the tumor cells. Indeed, harmful and lethal toxicity of chemotherapeutic agents, usually given by systemic routes, limits the dose of chemotherapy that can be injected. It is therefore essential to develop new delivery methods for antitumor therapy. Among them, electropermeabilization, also named electroporation, was successfully introduced in the 1970s [1,2]. The method is based on the transient permeabilization of the plasma membrane by applying a train of electric field pulses to the cells. It has been successfully developed for local antitumor drug delivery, a process called electrochemotherapy (ECT), providing direct delivery inside the cell of nonpermeant or low permeant drugs such as bleomycin and cisplatin [3,4]. Electrochemotherapy uses lower dosages of chemotherapeutic drugs than standard chemotherapy protocols. The ESOPE (European Standard Operating Procedures on Electrochemotherapy) study, a multi-institutional human clinical study on small skin tumor treatment by electrochemotherapy, established calibrated protocols. This study reported an objective response rate of 80% or more for the treatment of melanoma and non-melanoma nodules with ECT regardless of the drug used or the administration route [5]. Nowadays, electrochemotherapy is accepted in several European countries as a palliative treatment [6–8] and is also used in veterinary oncology in dogs, cats and horses [9]. In vivo electropermeabilization is also a promising method for the delivery of plasmid DNA for cancer gene therapy as shown with many preclinical and clinical trials [10–12].

The mechanisms supporting in vivo delivery of cytotoxic drugs, including strategies for overcoming obstacles for delivery, remain largely unknown although critical to the improvement and advancement of this therapeutic strategy [13]. While the processes of membrane electropermeabilization are still under discussion, there is a general agreement that the driving force of the structural changes is the applied field-induced transmembrane voltage. The transmembrane voltage, when higher than a threshold value, leads cell membranes to be transiently and locally permeable. Small molecules, such as anticancer drugs, enter the cell across caps of the plasma membrane facing the electrodes by a diffusion process [14]. In the vast majority of drug evaluation studies, monolayer cell cultures are used. However, these studies cannot predict the in vivo drug efficiency due to the inability of two-dimensional cultures to reproduce extracellular microenvironments and barriers, and also due to differences in cell phenotype between cells cultured as monolayers and cells in native tissue [15,16]. All these studies performed on cells in culture, although helpful, can therefore be of only limited use when relating to cells within a tissue environment. While some monolayer cultures produce extracellular matrix materials, they are incomplete and less dense than in a three-dimensional (3D) environment and thus present a less significant barrier for transport and cell-binding of delivered agents [17]. In order to more accurately predict and improve

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in vivo results of electrochemotherapy, to test new antitumor candidates, it is therefore desirable to study the electrotransfer effects in cells cultured in 3D models that mimic in vivo conditions.

The aim of the present study was to understand the drug delivery processes and cytotoxic effects that occur during electrochemotherapy in a tissular context. Our strategy was to visualize the delivery and to quantify the associated lethal effects of antitumor drugs in a 3D multicellular tumor spheroid (MCTS). To validate our model, we used non-permeant (bleomycin), low permeant (cisplatin) and permeant (doxorubicin) antitumor drugs, widely used in vivo in human clinical treatment. Upon growth, MCTS display a gradient of proliferating cells [18]. These proliferating cells are located in the outer cell-layers and quiescent cells are located more centrally. This cell heterogeneity is similar to that found in avascular microregions of tumors [19]. Moreover, as in tissues, tumor spheroids were found to contain extracellular matrix components such as collagens, laminin, fibronectin and glycosaminoglycans [20–22]. MCTS can therefore be used to evaluate tumor response to therapeutic agents [23]. Moreover, in the case of plasmid DNA electrotransfer and expression, MCTS have recently been reported to be much more relevant to an in vivo situation than cells in monolayers [24,25].

In the present study, we visualized, using different microscopy tools, the effect of the combination of electric pulses and drug delivery on the permeabilization of MCTS cells and the consequences in terms of growth and survival. Experiments were performed by using a small fluorescent molecule as a tool to detect cell permeabilization: propidium iodide (PI; 668 Da). Bleomycin, cisplatin and doxorubicin were used as cytotoxic drugs to assess the effect of electrochemotherapy. Bleomycin is a non-permeant antineoplastic antibiotic isolated from *Streptomyces verticillus* [26]. Its cytotoxicity results from its ability to make single and double strand DNA breaks in mammalian cells [27]. Cisplatin is a low-permeant platinum-based antineoplastic agent [28]. Its cytotoxic mode of action is mediated by its interaction with DNA to form DNA adducts, inducing intrastrand cross-links. Bleomycin and cisplatin are nowadays widely used for electrochemotherapy of solid tumors in cancer treatment, especially for cutaneous and subcutaneous tumors [29–31]. Doxorubicin, also called adriamycin, is a permeant antibiotic and a potent anticancer drug that inhibits reverse transcriptase and RNA polymerase and intercalates in DNA. Its molecular weight (580 Da) is intermediate between those of cisplatin (300 Da) and bleomycin (1400 Da). We took advantage of its endogenous fluorescence (excitation 470 nm, emission 592 nm) to observe its penetration and location inside the spheroid 3D model. The electric pulse parameters were those used clinically in electrochemotherapy protocols [5,32]. These experiments enabled us to address the following questions: (i) Can all the cells in a spheroid be efficiently permeabilized by the electric pulses? (ii) To what extent does their location affect that process? (iii) Are there effects of ECT on cell cohesion, growth and survival? and (iv) To what extent is the spheroid model relevant to an in vivo situation in terms of cytotoxic drug delivery and cell death?

2. Materials and methods

2.1. Chemicals

Cis-diamminedichloroplatinum (II) (Cisplatin, Cispt), doxorubicin hydrochloride (Doxo), propidium iodide (PI), formalin solution and Hoechst 33258 were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Bleomycin sulfate (Bleo) was purchased from Merck-Millipore (Molsheim, France).

2.2. Cell culture

The HCT-116 cell line (ATCC #CCL-247) originated from a human colorectal carcinoma. These cells were chosen for their ability to form multicellular tumor spheroids. HCT-116 cells were grown in Dulbecco's Modified Eagles Medium (Gibco-Invitrogen, Carlsbad, CA, USA) containing 4.5 g/l glucose, L-glutamine and pyruvate, supplemented with 10% (v/v) 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.

2.3. Generation of multicellular tumor spheroids (MCTS)

The method used to produce the spheroids was adapted from Kelm et al., and generate spheroids with a homogeneous size distribution of similar diameter [33]. Briefly, 20 μl drops containing 500 cells were placed on the lid of agar coated 48-well dishes containing 250 μl of culture media. The drops hanging from the lid of the dish allowed for the sedimentation of the cells and favored their aggregation. After 72 h the time required for cell aggregation the spheroids were transferred to the agar-coated bottom of the well containing 250 μl of culture medium. Multicellular spheroids were then allowed to grow for 4 to 7 more days and the spheroids used for experiments ranged from 7 to 10 days of age. The diameter of the spheroids was less than 600 μm preventing the appearance of a necrotic core with apoptotic cells.

2.4. Electropulsation and electropermeabilization of MCTS

MCTS to be electropermeabilized were suspended 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) between two flat parallel stainless steel electrodes (1 cm length, 0.4 cm width). Electropulsation was achieved using a CNRS Cell Electropulsator (Jouan, St Herblain, 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 1300 V/cm electric field intensity at room temperature. The electrical field intensity is defined as the ratio between voltage and inter-electrode distance. To visualize permeabilization, this electropulsation was performed after a 5 min incubation in the presence of propidium iodide (PI) at 2 μM. PI permeabilization was observed by confocal imaging (LSM 510 confocal microscope, Zeiss, Le Pecq, France) directly on live MCTS or on fixed frozen sections of MCTS. PI excitation was realized with a helium–neon laser set at 543 nm wavelength and the emitted light was collected through a 560–615 nm band pass filter. To mimic electrochemotherapy and study its cytotoxic effects on MCTS, electropulsation was carried out in the presence of 0.25 mg/ml of cisplatin (833 μM) or 0.25 U of bleomycin (1 mM) which are doses lower than those used in clinical treatments [29,31], or 2 μM of doxorubicin. Incubation time was 5 min at room temperature followed by application of the electric pulses or not. The macroscopic morphology of fresh MCTS was studied with a Leica macrofluo microscope coupled to a coolSNAP HQ camera (Roper Scientific, USA).

2.5. Fixation and cryo-sectioning of MCTS

When necessary MCTS were fixed in 10% formalin solution at room temperature for 2 h, then placed in phosphate-buffered saline (PBS) 10% sucrose at 4 °C for 2 h, and finally overnight in PBS 30% sucrose at 4 °C. MCTS were then embedded in optimal cutting temperature compound (OCT, Electron Microscopy Sciences, Hatfield, USA) and stored at −80 °C. Sections were cut 10 μm thick. Surface plots were obtained using Image J software (NIH, Bethesda, USA). They display a graph of the surface distribution of the intensities of pixels on the entire corresponding cryosection pictures.

2.6. MCTS growth curve

MCTS growth was followed by taking photographs of the spheroids over five days with a Leica macrofluo microscope coupled to a coolSNAP HQ camera. The size of the projected area of the MCTS on each image