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Nanosecond pulsed electric field (nsPEF) enhance cytotoxicity of cisplatin to hepatocellular cells by microdomain disruption on plasma membrane

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ABSTRACT

Previous studies showed nanosecond pulsed electric field (nsPEF) can ablate solid tumors including hepatocellular carcinoma (HCC) but its effect on cell membrane is not fully understood. We hypothesized nsPEF disrupt the microdomains on outer-cellular membrane with direct mechanical force and as a result the plasma membrane permeability increases to facilitate the small molecule intake. Three HCC cells were pulsed one pulse per minute, an interval longer than nanopore resealing time. The cationized ferritin was used to mark up the electronegative microdomains, propidium iodide (PI) for membrane permeabilization, energy dispersive X-ray spectroscopy (EDS) for the negative cell surface charge and cisplatin for inner-cellular cytotoxicity. We demonstrated that the ferritin marked-microdomain and negative cell surface charge were disrupted by nsPEF caused-mechanical force. The cell uptake of propidium and cytotoxicity of DNA-targeted cisplatin increased with a dose effect. Cisplatin gains its maximum inner-cellular cytotoxicity when combining with nsPEF stimulation. We conclude that nsPEF disrupt the microdomains on the outer cellular membrane directly and increase the membrane permeabilization for PI and cisplatin. The microdomain disruption and membrane infiltration changes are caused by the mechanical force from the changes of negative cell surface charge.

1. Introduction

Nanosecond pulsed electric field (nsPEF) is a novel loco-regional tumor ablation methodology with high voltage, low energy and non-thermal effects [1,2]. The previous studied have found nsPEF can ablate different solid tumors [3–7] in mouse [8,9], rat [10] and human [11,12] by causing autophage [13], cell apoptosis [14], immune stimulation [8,15,16] and metastasis inhabitation [16]. But how nsPEF affect the plasma membrane is not fully understood. Some studies suggest nanopore formation on the nsPEF-treated cell membrane [17,18]. The nanopores have small pore size (<2 nm), quite different from the large pores (>2 nm) in electroporation stimulated by longer pulses (micro- and millisecond pulsed electric fields) [19].

The plasma membrane consists of microdomains which is smaller than 50 nm in diameter [20]. microdomains in lipid systems are well studied and have been shown to be important to a variety of new technologies [21]. They are lipid-rich and tend to be dynamic but homogenous. When temperature, pressure or ionic strength changes, the microdomains can be disrupted and then become heterogeneous [22,23]. So it can be used as marker to reflect the mechanical force on the cell surface. In this study we focused on the electronegative microdomains outside the cell membrane and demonstrated that other than nanopores or electroporation, nsPEF disrupt the charged particles on the cell membrane and cause the redistribution of microdomains. As a result, the cell membrane permeability to PI it increased. The small molecule such as chemotherapy drugs can infiltrate cells..

2. Materials and methods

2.1. Cell culture

Three human hepatocellular carcinoma cell lines were treated

Abbreviations: HCC, hepatocellular carcinoma; nsPEF, nanosecond pulsed electric field; PI, propidium iodide; EDS, energy dispersive X-ray spectroscopy

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by nsPEF. The SMMC7721and BEL7402 cells were purchased from China Infrastructure of Cell Line Resources (Shanghai, China). High metastatic HCC cell line HCCLM3 was purchased from the Liver Cancer Institute, Zhongshan Hospital, Fudan University. All cells were grown in RPMI-1640 (Gibco, Life Technologies, Grand Island, USA) containing 10% fetal bovine serum(Gibco, Life Technologies, Grand Island, USA), penicillin (100 units/ml, Sigma, Missouri, USA), and streptomycin (0.1 mg/ml, Sigma, Missouri, USA) in surface cell culture dish (Corning, New York, USA) and cultured at 37 °C in a humidified incubator containing 5%/95% CO_2/air (Froma SeriesIIWater Jacket CO_2 Incubator, Thermo Scientific, MA, USA).

2.2. Pulse treatment solution (PTS)

Single-Cell suspensions were prepared with PBS buffered 0.25% Trypsin (Sigma, W/V) and 0.02% EDTA (W/V) and then suspended in pulse treatment solution, which is a Ca^{2+} and Mg^{2+} free phosphate buffered PH7.2–7.4(measured by Sartorius PB-10) solution and rectified to 310 mOsm/L and about 70 Ω cm electrical resistivity with sodium chloride and glucose based on RPMI-1640.

2.3. Pulse generator and treatment parameters

A typical Blumlein line pulse generator for analyzing nsPEF effects on suspended cells were configured with a pulse-forming network (Fig. 1). Cells were put into a cuvette (Bio-Rad BTX). Pulses were applied at 40 kV/cm, one pulse per minute, the wave form were measured by Tektronix DPO4054B Digital Phosphor Oscilloscope with high voltage probe (P6015A. Tektronix. USA) as described in the previous publications [7,16]. Typical nsPEF waveform in our study shows a voltage of 8 kV, 100 ns pulse width,15 ns fast rising time (Supplementary figure).

2.4. Propidium iodide (PI) uptake

Cells were put into tubes with 30 umol/L PI (Sigma, U. S. A.) in PBS at room temperature in the dark for 15 min for staining. Then washed and re-suspended for FACS analysis. (CantoIIBecton Dickinson, San Jose, CA),

Cationized ferritin marking on Membrane Well-mixed 0.1 ml cationized ferritin solution (10 mg/ml cationized ferritin in 0.15 mol/L NaCl, Sigma, U. S. A.) and 0.5 ml PH7.2 Veronal-HCl (0.05 mol/L baritone buffered saline) were added to 10^6 cells in 1 ml tube (0.01 mol/ml PH7.4 PBS). Then cells were fixed in 1 × PBS buffered 4% para-formaldehyde and 5% formaldehyde solution (v/v),1 × PBS buffered 2% para-formaldehyde and 2.5% glutaraldehyde solution (v/v), dehydrate and embed the with resin EP812(Hedebio, Beijing, China).

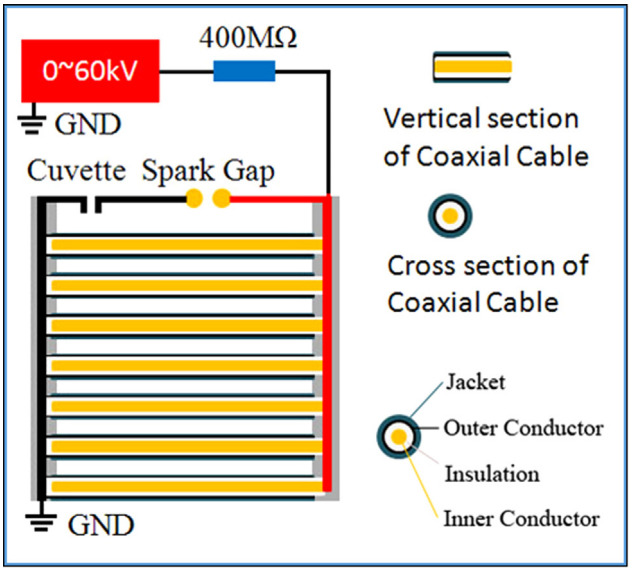

Fig. 1. The schematic diagram of nanosecond Pulse Generator. Blumlein electric pulse generator with high voltage DC resource was made by 7 paralleled cables to produce 100ns pulsed electric field. It was equipped with current limiting resistance, spark gap switch and cuvette adapter to facilitate the cell treatment

2.5. Transmission electron microscope

Cells were fixed with 2.5% glutaraldehyde and then 1% OsO4. After dehydration through a graded ethanol series and acetone, and three washes with 100% (v/v) acetone (30 min), the samples were embedded in Epon812. 100 nm ultra-thin sections were routinely processed with Ultramicrotome Leica EM UC7 (Wetzlar and Mannheim, Germany) and placed on 300 mesh nickel grids. Samples were analyzed with the help of a Hitachi-7650 (Hitachi, Japan).

2.6. The energy dispersive X-ray spectroscopy (EDS)

X-ray spectroscopy analysis were performed at Genesis XM2 (EDAX, NJ, USA) and the software of Genesis 4000(V3.61, EDAX, NJ, USA) with X-ray energy of 12.68 KV in a 180°scattering geometry. The focal spots with electron-dense of cellular membrane was positive sites; focal spots on cellular membrane with electron-translucent or intracellular sites were negative sites. The mean count of the peak of FeKα (Fe1), FeKβ(Fe2), NiKα(Ni1), NiKβ(Ni2) and that of background had been applied to calculate signal-to-noise ratio.

2.7. Cell viability analysis

CCK-8 was used to measure cell viability. Exponentially growing SMMC7721, BEL7402 and HCCLM3 cells (100 µl, 8×10^4 cells/ml) were seeded into 96-well plates (Corning Incorporated, Corning, NY, USA). Cells were cultured in RPMI-1640 medium containing 10% FBS at 37° and 5%CO_2 for 24 h,10 µl CCK-8 (Dojindo, Kumamoto, Japan) solution was added to each well, after another 3 h, the optical density was measured at an absorbent of 450 nm using a microplate reader (ELx800; BioTek Instruments, Inc., VT, USA). The relative cell survival rate was calculated by OD value divided by control.

2.8. Inhibition to cell proliferation of single and combined use of cisplatin and nsPEF

For Cisplatin inhibition, cells were detached with Trypsin and then dispensed into 96-well plates and incubated with Cisplatin containing 10%FBS RPMI-1640 (With 0, 2, 4, 8, 16, 32 mg/ml cisplatin in concentration respectively)for 24 h and then tested by CCK8. Mean ± the standard deviation (SD) were applied to calculate the half maximal inhibitory concentration (IC50). For nsPEF treatment, cells were detached and dispensed in cuvettes and treated with nsPEF(100 ns, 40 kV/cm for 0, 6, 12, 18, 24 and 30pulses). Cells were then seeded into 96-well plates and cultured for 24 h and then CCK8 assay performed to analyze the cell viability. Mean ± the standard deviation (SD) were applied to calculate the half maximal inhibitory Pulsing (IP50). For synergic effect of cisplatin and nsPEF, cells were detached and dispensed in cuvette and treated with nsPEF(100 nsof 40 kV/cm for 12 pulses). Then cells were then seeded into 96-well plates, 5 ug/ml cisplatin was added at the same time or after 2 h. After 24 h culture, cell viability was tested by CCK8 assay.