Intense picosecond pulsed electric fields induce apoptosis through a mitochondrial-mediated pathway in HeLa cells

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Abstract. The application of pulsed electric fields (PEF) is emerging as a new technique for tumor therapy. Picosecond pulsed electric fields (psPEF) can be transferred to target deep tissue non-invasively and precisely, but the research of the biological effects of psPEF on cells is limited. Electric theory predicts that intense psPEF will target mitochondria and lead to changes in transmembrane potential, therefore, it is hypothesized that it can induce mitochondrial-mediated apoptosis. HeLa cells were exposed to psPEF in this study to investigate this hypothesis. MTT assay demonstrated that intense psPEF significantly inhibited the proliferation of HeLa cells in a dose-dependent manner. Typical characteristics of apoptosis in HeLa cells were observed, using transmission electron microscopy. Loss of mitochondrial transmembrane potential was explored using laser scanning confocal microscopy with Rhodamine-123 (Rh123) staining. Furthermore, the mitochondrial apoptotic events were also confirmed by western blot analysis for the release of cytochrome C and apoptosis-inducing factor from mitochondria into the cytosol. In addition, activation of caspase-3, caspase-9, upregulation of Bax, p53 and downregulation of Bcl-2 were observed in HeLa cells also indicating apoptosis. Taken together, these results demonstrate that intense psPEF induce cell apoptosis through a mitochondrial-mediated pathway.

Introduction

Targeted non-invasive treatment of tumors is the most promising area of medical research worldwide. The application of pulsed electric fields (PEF) is emerging as a new technique for tumor therapy. According to the pulse duration, PEF can be classified into millisecond (msec), microsecond (µsec), nanosecond (nsec) and picosecond (psec). Most researchers have focused on the millisecond, microsecond and nanosecond pulse range for a more in-depth study.

Weaver noted that the lipid bilayer of cells is temporarily rearranged, followed by the formation of aqueous channels in the cell membrane when exposed to long pulses (msec to µsec), called electroporation (1). These pulses cause reversible electrical breakdown (REB), accompanied with a tremendous increase in molecular transportation across the cell membrane; thus, many electroporation techniques are applied in cell transfection for gene expression and drug delivery. Okino et al (2) first originated the concept of electrical chemotherapy (ECT) on the basis of electroporation. Hofmann et al (3) and Dev et al (4) applied ECT together with administration of bleomycin for the treatment of tumors. The drug was able to kill the cancer cells effectively at a relatively low concentration with minimal systematic side effects. Although ECT may enhance the delivery of drugs, it is still not able to directly kill tumor cells and negate their side effects. Yet, if the electric field strength continues to increase, the pores in the cell membrane enlarge, causing a loss of membrane intactness and the direct killing of cancer cells (5). This phenomenon is termed irreversible electrical breakdown (IREB). Miller et al (6) and Rubinsky et al (7) demonstrated that with proper parameters, IREB could completely ablate human hepatocarcinoma cells (HepG2) and prostate cancer cells in vitro without inducing thermal damage. As the pulse duration decreases to nanoseconds, this leads to intracellular electromanipulations such as apoptosis, intracellular calcium burst, cytoskeleton, nuclear membrane, DNA and telomere damage, with the outer membrane remaining intact. Thus, this technique may be used in tumor treatment and gene therapy (8-14). Most recently, it has been shown that such PEF caused shrinkage and even complete elimination of melanoma tumors (15).

However, the application of millisecond, microsecond or nanosecond PEF requires the use of an invasive or minimally invasive needle or plate electrodes, to guide the puncture of tumor tissue, which to some extent limits the clinical application of this method. Picosecond PEF (psPEF) has a wealth

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of ultra-broadband spectrum, with extended time and spatial resolution, and low signal distortion. It could be transferred to target deep tissue non-invasively and precisely with wide-band antennas (16,17). Yet, research on the biological effect of psPEF on cells is limited. Electric theory predicts that intense psPEF will target mitochondria and lead to changes in transmembrane potential, therefore it is hypothesized that it may induce cell apoptosis through the mitochondrial pathway.

Our group has dedicated its study of the antitumor effects of psPEF or nsPEF for many years. In this study, we tested the hypothesis that intense psPEF induces cell death through mitochondrial apoptosis. HeLa cells were exposed to psPEF. Our study included three steps: to investigate i) the dose-effect of psPEF on cells, ii) the morphology of apoptosis and iii) the mechanisms of mitochondrial apoptosis.

Materials and methods

Cell culture. HeLa, a human cervical carcinoma cell line was obtained from the Institute of Ultrasound Engineering in Medicine of Chongqing Medical University. Cells were cultured in RPMI-1640 medium (Hyclone, USA) supplemented with 10% fetal calf serum (Amresco, USA), streptomycin (100 IU/ml) and penicillin (100 IU/ml) at 37°C in a 5% humidified CO₂ incubator. The cells were fed until reaching 50-75% confluence, expanded by 0.25% trypsin (Hyclone, USA) and subcultured at lower numbers in new culture flasks.

Picosecond pulsed electric field (psPEF) treatment. Cells were harvested with trypsin and re-suspended in fresh RPMI-1640 medium to a concentration of 2x10⁵ cells/ml. Cells loaded into cuvettes and merely placed into the circuit without being pulsed were used as the normal controls. A total amount of 100 µl of cell suspension was placed in cuvettes and exposed to 800 psec pulses with an electric field amplitude of 250 kV/cm. In the MTT assay, the quantities of pulse numbers were from 100 to 5000, and in other tests, the groups were divided by the quantities of pulse numbers (group A, normal control; group B, 1000 pulses; group C, 3000 pulses; and group D, 5000 pulses).

MTT assay. The cell viability was investigated using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. This assay was performed in quintuplicate. After treatment, HeLa cells were seeded in 96-well plates (5000 cells/well-1200 µl) and routinely cultured in an incubator for 6, 12, 24 and 48 h. A normal control and a blank group (without cells) were also included. After incubation, 20 µl MTT (5 mg/ml) (Amresco) was added to each well, and the plates were incubated for another 4 h. Following incubation, the culture medium was removed by gentle aspiration and replaced with 150 µl DMSO, and the plates were agitated for 10 min to dissolve the formazan crystals. Then, the optical density of the 96-well culture plates was measured using an enzyme-linked immunosorbent assay (ELISA) reader at 490 nm.

Transmission electron microscopy (TEM) analysis. After treatment with 5000 pulses, the cells were harvested and grown in RPMI-1640 medium containing 10% SFS for 12 h. Floating cells were then harvested together with adherent cells and centrifuged at 200 x g for 5 min. The cell pellets were fixed overnight in a 0.2 M sodium cacodylate buffer solution (pH 7.4) containing 2% glutaraldehyde at 4°C. Samples were then post-fixed in cacodylate-buffered 1% osmium tetroxide, dehydrated, and embedded in Epon 812 (Structure Probe, Inc., West Chester, PA, USA) for ultra-thin sectioning. The ultra-thin sections were stained with uranyl acetate and lead citrate and viewed with a Hitachi-7500 transmission electron microscope (Hitachi, Japan) at the Chongqing Medical University Cell Imaging Facility.

Measurement of mitochondrial membrane potential (Δψm). Δψm was measured by laser scanning confocal microscopy (LSCM) (Leica TCS-SP2, Germany) using the cationic lipophilic green fluorochrome Rhodamine-123 (Rh123) (Molecular Probes) (Sigma, USA). After treatment with pulses, the cells were routinely cultured in an incubator for 6 and 12 h. After incubation, they were harvested, washed, resuspended in medium containing Rh123. Then the cells were incubated at 37°C in an CO₂ incubator for 15 min. The cells were washed again and resuspended in medium. Finally, the cells were subjected to LSCM analysis by detecting the green fluorescence signals.

Western blot analysis. After treatment with pulses, the cells were harvested and lysed in ice-cold RIPA cell lysates (CST, USA). Lysates were centrifuged at 12,000 x g for 20 min at 4°C, and the protein content was measured with the BCA Protein Assay kit (Sangon Biotech, China). Thirty micrograms of protein was mixed with sodium dodecyl sulfate (SDS) (Sigma) sample buffer, denatured by boiling, and separated on 12% SDS-polyacrylamide gels. Proteins were then electroblotted to nitrocellulose membranes (Millipore, USA) and blocked for 2 h at room temperature in PBS buffer containing 5% non-fat milk. Membranes were then incubated overnight at 4°C with the respective primary antibodies. Antibodies against cytochrome C (Cyt c) and apoptosis-inducing factor (AIF) were purchased from CST. Anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase (CST) was used to visualize the stained bands with an enhanced chemiluminescence (ECL) visualization kit. Equal loading of protein was confirmed by stripping the blots and reprobing with β-actin antibody.

Real-time polymerase chain reaction (PCR). Total RNA was extracted from treated cells by using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. Reverse transcription of 2 µg of RNA was performed using the reverse transcription kit, M-MLV1 (Promega, China). The PCR reactions were carried out using intercalation of SYBR Green master mix following the manufacturer's protocol (Applied Biosystems, USA 7900HT Fast Real-Time PCR System). Equal amounts of cDNA, as determined by detection of the fluorescence signals, were used to quantify the expression of caspase-3, Bax, Bcl-2 and p53 genes. The following primers were used: caspase-3 forward, 5'-CGTGATTGTGTCATCCTCAC3'- and reverse, 5'-CCATCATTGGTTAATGCTC3'; Bax forward, 5'-GACGAACTTGCAGAATGTCAT-3' and reverse, 5'-AGGAAAGTCTCATCGTCGTC3'; Bcl-2 forward, 5'-CAGGGAGAACAGGTTGATA3'- and reverse, 5'-CAGGCTGGAAGGAGAAT3'; p53 forward,
5'-CAGTCTACCTCCCGCCATA-3' and reverse, 5'-GCAAGCAAGGGTTCAAAG-3'. Reactions were performed in duplicate from three separate RNA preparations.

Statistical analysis. Statistical analyses were performed using SPSS for Windows 13.0. Data are presented as the mean ± SD, and were subjected to analysis using one-way ANOVA, followed by LSD t-test for multiple comparisons among groups. A probability value of P<0.05 was defined as statistically significant.

Results

Growth inhibition of HeLa cells. To determine cell viability after psPEF treatment, HeLa cells were exposed to 0-5000 pulses of 250 kV/cm amplitude. Growth inhibition was determined by MTT assay. The cell survival rate in normal control cells was taken as 100% viability. The percentage of cell inhibition was determined as the (absorbance of normal control cells - absorbance of treated cells/absorbance of normal control cells - absorbance of blank group) x 100%. MTT assay demonstrated that psPEF inhibited the growth of HeLa cells in a dose-dependent manner (Fig. 1). For each line, the cell inhibition rate increased in parallel with the number of pulses, while it significantly increased from 500 pulses (P<0.05). We evaluated the growth inhibition at 6, 12, 24 and 48 h post-pulses, and the result showed that at a given number of pulses, psPEF achieved a plateau of maximum cell inhibition at 12-h post-pulses.

Ultrastructural observation. TEM was used to characterize the ultrastructural changes in the HeLa cells in response to psPEF exposure. TEM observation showed that normal HeLa cells were intact with well-distributed chromatin and clear nuclear membrane (Fig. 2A). However, in response to psPEF exposure (5000 pulses), the cells changed to a shrunken state with an intact membrane, aggregated chromatin and pseudopodia-like protrusions which suggested apoptosis (Fig. 2B).

psPEF induce depolarization of mitochondrial membrane potential (Δψm). Depolarization of Δψm is associated with a lack of Rh123 retention and a decrease in fluorescence. The value was measured by LSCM using Rh123. Fig. 3 shows the fluorescence images of the control and treated groups 6 h after the pulses. Obviously, the green fluorescence intensity thinned down after the pulses, which indicates that Δψm decreased. Fluorescence images 12 h after the pulses were also obtained (data not shown). Fluorescence intensity is shown in Table I. Fluorescence intensity in the treated groups was significantly lower compared to that in the control (all P<0.01). The minimum fluorescence intensity was observed after 6 h, and no further decreases were observed thereafter.

psPEF induce cytochrome C (Cyt c) and apoptosis-inducing factor (AIF) release. Under physiological conditions, these two proteins are located in the mitochondrial intermembrane space. In the process of mitochondrial apoptosis, they are released from mitochondria to the cytoplasm. Western blotting
analysis was used to quantify the expression of Cyt c and AIF in the cytoplasm. β-actin served as a control for sample loading. As a result, we found that Cyt c and AIF accumulated 6 h after treatment with psPEF. The protein levels were significantly higher after pulsing (P<0.05).

**psPEF generate activation of caspase-3 and caspase-9.** Caspase-3 and caspase-9 are involved in the process of mitochondrial pathway-mediated apoptosis. In response to apoptotic stimuli, they become activated. Real-time PCR was performed to quantify the altered expression of caspase-3 and caspase-9 (Fig. 5).

**Table I. Fluorescence intensity of HeLa cells by LSCM.**

|          | A          | B          | C          | D          |
|----------|------------|------------|------------|------------|
| 6-h      | 36.24±7.23 | 50.32±7.43 | 74.31±10.28| 94.13±12.39|
| 12-h     | 39.14±5.75 | 52.40±8.67 | 75.90±4.97 | 91.72±10.78|

LSCM, laser scanning confocal microscopy. Group A, normal control; group B, 1000 pulses; group C, 3000 pulses; and group D, 5000 pulses. aCompared with group A, P<0.01; bcompared with the 6-h group, P>0.05.
and caspase-9. Activation of caspase-3 and caspase-9 was observed. Each treated group showed significant difference in comparison to the control group (P<0.05) (Fig. 5).

**Discussion**

Cervical cancer is one of the most common gynecological malignancies. Its incidence in young women has increased in recent years (18). The traditional surgical treatment often leads to severe damage of the genital tract and affects the sexual function and fertility of patients. Despite advances in surgical techniques, including conservative treatment such as radical tracheectomy, the fertility of patients is still highly affected (19). Non-invasive treatment with preserved fertility is the expectation for both doctors and patients.

Pulsed-electric field is a new biomedical engineering technique which can be used as electrochemotherapy, tumor ablation and intracellular electromanipulation. Whereas studies on the effects of millisecond, microsecond or nanosecond PEF have already led to medical applications, to our knowledge there are few experimental studies on the biological effects of psPEF.

The electric field possesses parameters related to different biophysical effects, that is, the impact of electric field pulses on cells has a certain window effect (20,21). When the pulse duration is used as a reference point, the corresponding changes in the biological effects caused by different parameters of pulses are shown in Fig. 7. Millisecond or microsecond PEF mainly target the outer membrane, and there is little influence to the cell nucleus, mitochondria and other organelles; thus, it causes electroporation to the outer membrane. As the pulse duration decreases, the electroporation effect changes gradually from the outer membrane to the intracellular organelle membrane. Submicrosecond PEF is capable of inducing significant voltages across both the inner and outer membranes, therefore, causing damage to both the inner and outer membranes.

While these effects of PEF continue to be explored, a new domain of pulsed electric field interactions with cell structures and functions unfolds when the pulse duration is reduced to values such that membrane charging becomes negligible.
For mammalian cells, this holds for a pulse duration of one nanosecond or less (22). We dare to assume from the rules above, that when the electrical pulse duration is shorter than one nanosecond, PEF are able to induce larger voltage across the inner membrane and to act mostly on intracellular substructures. According to cell biology and electromagnetic theory, the mitochondrial membrane charging time constant (a few hundreds of picoseconds) is much shorter than the nuclear membrane and the cell membrane charging time constant (tens of nanoseconds, and hundreds of nanoseconds, respectively) (23,24). Under the action of intense psPEF, the mitochondrial membrane will charge rapidly, at a time when the nuclear membrane and the cell membrane have no chance to respond. Thus, the mitochondrial transmembrane potential will be altered. We speculate that intense psPEF target the mitochondria and lead to changes in transmembrane potential, release of Cyt c and AIF, activation of caspase 9 and caspase 3, and finally apoptosis.

Schoenbach et al. applied subnanosecond pulses to B16 (mouse melanoma) cells. Initial experiments at a 800-psec pulse width and an extremely high electric field of 950 kV/cm showed that with a relatively small number of pulses, a considerable increase in caspase activation, externalization of phosphatidyl serine and programmed cell death was initiated (22). For pulse amplitudes of 550 kV/cm, approximately 50% of the cells absorbed trypan blue immediately after pulsing, whereas only 20% absorbed it after 1 h. This indicated that the plasma membrane in a majority of the cells affected by the pulses recovered with a time constant of approximately 1 h. The cells that exhibited trypan blue uptake after this time suffered cell death through apoptosis (25). Experiments where platelets were exposed to 150-psec pulses with an electric field of 150 kV/cm indicated a pulse-number-dependent uptake of calcium (26).

In the present experiments we demonstrated the response of HeLa cells to psPEF. Our results revealed that psPEF is capable of inducing cell apoptosis through a mitochondrial-mediated pathway. Firstly, MTT assay demonstrated that intense psPEF significantly inhibited the proliferation of HeLa cells, and typical characteristics of apoptosis in the HeLa cells were observed under TEM. Proteins located in the inner mitochondrial membrane, such as Cyt c and AIF, play an important role in apoptosis. Once Cyt c enters the cytoplasm, it conjugates with apoptotic peptide activating factor 1 (ApaF-1) and facilitates the activation of procaspase-9, which finally activates caspase-3. Thus, the release of Cyt c from the mitochondria into the cytoplasm triggers apoptosis via the caspase-3-dependent pathway. AIF is a key trigger of caspase-independent apoptosis. Western blot analysis showed that Cyt c and AIF significantly increased in the cytoplasm after exposure to psPEF compared to the untreated groups, and loss of Δψm was observed by LSCM. This suggests that both the caspase-dependent and -independent apoptotic pathways are involved in psPEF-induced apoptosis in HeLa cells through the mitochondrial pathway. The Bcl-2 family members and the p53 gene are key regulators of the mitochondrial pathway of apoptosis. Bcl-2 and Bax are the best characterized proteins of the Bcl-2 family, and the Bax/Bcl-2 ratio is a key factor to determine cell death after exposure to death stimuli. Elevated expression levels of caspase-3, caspase-9, Bax, p53 and reduced levels of Bcl-2 in the treated groups were observed in comparison to the normal group indicating apoptosis.

In summary, the present data provide evidence that psPEF induce apoptosis in cultured human cervical cancer cells, and the apoptotic effect is possibly through the mitochondrial-mediated pathway. The use of picosecond pulses not only allows us to enter a new field of field-cell interactions, but it may open the door to a range of noninvasive therapeutic applications. Further studies are needed to elucidate the cell responses to psPEF in detail.

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