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Different involvement of extracellular calcium in two modes of cell death induced by nanosecond pulsed electric fields

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ABSTRACT

Exposure of cultured cells to nanosecond pulsed electric fields (nsPEFs) induces various cellular responses, including the influx of extracellular Ca^{2+} and cell death. Recently, nsPEFs have been regarded as a novel means of cancer therapy, but their molecular mechanism of action remains to be fully elucidated. Here, we demonstrate the involvement of extracellular Ca^{2+} in nsPEF-induced cell death. Extracellular Ca^{2+} was essential for necrosis and consequent poly(ADP-ribose) (PAR) formation in HeLa S3 cells. Treatment with a Ca^{2+} ionophore enhanced necrosis as well as PAR formation in nsPEF-exposed HeLa S3 cells. In the absence of extracellular Ca^{2+}, HeLa S3 cells were less susceptible to nsPEFs and exhibited apoptotic proteolysis of caspase 3 and PARP-1. HeLa S3 cells retained the ability to undergo apoptosis even after nsPEF exposure but instead underwent necrosis, suggesting that necrosis is the preferential mode of cell death. In K562 and HEK293 cells, exposure to nsPEFs resulted in the formation of necrosis-associated PAR, whereas Jurkat cells exclusively underwent apoptosis independently of extracellular Ca^{2+}. These observations demonstrate that the mode of cell death induced by nsPEFs is cell-type dependent and that extracellular Ca^{2+} is a critical factor for nsPEF-induced necrosis.

Introduction

Pulsed electric fields (PEFs) are utilized for a broad range of applications in life sciences, because they have various biological effects depending on the duration and intensity of electric pulses. Milli-to-microsecond PEFs are widely exploited in the introduction of macromolecules into living cells because these PEFs transiently generate pores on the cell membrane and achieve electrotransfer of macromolecules, such as plasmid DNA and anti-tumor drugs [1–3]. Ultrashort PEFs in the range of nanoseconds (nanosecond pulsed electric fields, nsPEFs[1]) have distinct effects on cells. Exposure of human cells to nsPEFs does not result in the formation of pores suitable for macromolecule transfer. Instead, nsPEFs generate small pores (nanopores) that allow ion permeation across the cell membrane [4–6]. Consistent with nanopore formation, nsPEFs have been reported to provoke a transient increase in cytoplasmic Ca^{2+} levels, primarily by causing an influx of extracellular Ca^{2+} [7–9].

In addition to the Ca^{2+} influx, nsPEFs elicit multiple cellular responses that vary depending on the intensity of the applied electric fields. Relatively mild nsPEFs induce intracellular signaling responses, including sequential phosphorylation of proteins in the MAPK pathways and expression of immediate-early genes such as *c-fos* and *c-jun* [10,11]. Furthermore, the AMPK pathway is activated by mild nsPEFs in an extracellular Ca^{2+}-dependent manner, indicating that extracellular Ca^{2+} affects intracellular events induced by nsPEFs [12]. Sublethal nsPEFs provoke stress responses that involve phosphorylation-mediated intracellular signaling and translational suppression [13]. Exposure to intense nsPEFs results in cell death *in vitro* [14–17] and tumor regression *in vivo* [18–20], suggesting the therapeutic potential of intense nsPEFs for cancer. Both apoptotic and necrotic cell death can be induced in cultured cell lines after nsPEF exposure [16,17,21]. Similarly, *in vivo* studies have demonstrated the coexistence of apoptotic and non-apoptotic cell death in tumors exposed to nsPEFs [19,22].

Apoptosis and necrosis are the major forms of cell death and are characterized by discrete molecular events [23,24]. Apoptosis is initiated by various stimuli and executed by activated effector caspases [25,26]. Caspase 3 is a major effector caspase and plays a critical role in the manifestation of apoptotic features [27,28]. Caspase 3 exists in an inert form under normal physiological conditions and is activated by proteolysis yielding the cleaved forms of

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[1] *Abbreviations used:* nsPEF, nanosecond pulsed electric field; PAR, poly(ADP-ribose); PARP-1, poly(ADP-ribose) polymerase-1.

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caspase 3, which are widely considered to be a reliable marker for apoptosis execution [27,28]. Necrosis is another form of cell death and is elicited by serious physiological perturbations [29,30]. Although necrosis has been regarded as an uncontrolled passive process for a long time, recent studies have clearly demonstrated that elaborate mechanisms underlie some forms of necrosis [30]. Necrotic cells frequently exhibit massive poly(ADP-ribose) (PAR) formation [31–33] that is detectable as a smear in western blotting using anti-PAR antibody. PAR formation is primarily carried out by poly(ADP-ribose) polymerase-1 (PARP-1), which is one of the major substrates of activated caspase 3 in apoptotic cells [34,35]. Thus, the following biochemical events are used to discriminate between apoptotic and necrotic cell death; apoptotic cells exhibit proteolytic cleavage of caspase 3 and PARP-1, and necrotic cells display massive PAR formation with no sign of apoptosis-associated proteolysis.

Recently, nsPEFs have received considerable attention as a novel means for cancer therapy, because of the effectiveness of nsPEFs in killing tumor cells [14–16,18–22]. However, the molecular mechanisms of nsPEF-induced cell death remain incompletely understood. In particular, little information is available on the involvement of extracellular Ca^{2+} in nsPEF-induced cell death. In this study, we analyzed the modes of cell death induced by nsPEFs in relation to extracellular Ca^{2+}. We demonstrated that extracellular Ca^{2+} significantly affects the choice of cell death modes and cell viability in nsPEF-exposed HeLa S3 cells. Furthermore, we found that one of two cell death modes is induced after nsPEF exposure in a cell-type dependent manner. These findings provide novel insights into nsPEF-induced cell death and a mechanistic rationale for the use of nsPEFs in cancer therapy.

Materials and methods

Generation of nsPEFs

Generation of nsPEFs was performed as described previously [10,13]. Briefly, the electronic system for the generation of nsPEFs was designed by Kumamoto University and manufactured by Suematsu Electronics Co., Ltd. (Pulsed Power Modulator MPC3000S, Kumamoto, Japan). Voltage waveforms of the electric pulses were monitored using a P6015A high voltage probe and a DPO4054 digital phosphor oscilloscope (Tektronix). The wave form of nsPEFs under our standard experimental conditions has been described previously [10]. The average pulse width at half maximum was estimated to be approximately 80 ns [10].

Cell culture and exposure of cells to nsPEFs

HeLa S3 and human embryonic kidney 293 (HEK293) cells were grown in α-minimum essential medium (αMEM) supplemented with fetal bovine serum (FBS) and penicillin/streptomycin. Jurkat, HL-60, and K562 cells were cultured in RPMI-1640 medium supplemented with FBS and penicillin/streptomycin.

Prior to exposure to nsPEFs, attached cells were washed twice with Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (D-PBS) and gently detached from culture dishes by treatment with 1 mM EDTA in D-PBS. Cells were collected by brief centrifugation and resuspended in appropriate media. To prepare Ca^{2+}-free medium, Dulbecco's modified Eagle's medium (DMEM) without Ca^{2+} (Invitrogen) was supplemented with dialyzed FBS (Sigma–Aldrich), from which Ca^{2+} was removed by dialysis against 0.15 M NaCl with a cut off value of 10,000 Da. For the preparation of Ca^{2+}-containing medium, $CaCl_2$ was added to the Ca^{2+}-free medium at 1.8 mM, because commonly used media such as αMEM and DMEM contain Ca^{2+} at this concentration.

The cell suspension (400 μl) was placed in an electroporation cuvette that contained a pair of parallel aluminum electrodes with a 4 mm-gap (#5540, MβP, Thermo Fisher Scientific). nsPEFs at 1 Hz were generated as described above and applied to the cell suspension in the cuvette. The cells were then immediately diluted 5-fold into pre-warmed media and incubated at 37 °C.

Measurement of cell viability and statistical analysis

Cells were exposed to nsPEFs as described above and incubated at 37 °C for 24 h. Cell viability was measured by the MTT method with a Cell Proliferation Kit I (Roche Applied Science) according to the manufacturer's procedures. Average values and standard deviations were calculated from five independent experiments. Statistical analysis was performed using a Student's *t*-test, and significance levels (p value) are indicated in the figures.

Western blotting

Cells were pelleted by centrifugation and lysed in SDS–PAGE loading buffer that contained 1% SDS. After incubation at 100 °C for 10 min, cell lysates were sonicated with a microsonicator (Model UR-20P, Tomy Seiko). After brief centrifugation, the lysates were subjected to SDS–PAGE followed by electrotransfer to a PVDF membrane. Proteins of interest were reacted with specific primary antibodies and detected by the chemiluminescence method as described previously [10]. Primary antibodies against PARP-1 and caspase 3 were obtained from Cell Signaling Technology. A monoclonal antibody for PAR (clone 10H) was purchased from Tulip BioLabs. An antibody against Ku80 was a kind gift from Dr. David Chen (University of Texas Southwestern Medical Center).

Results

Extracellular Ca^{2+} is required for PAR formation by nsPEFs

Several studies have shown that nsPEFs cause a rapid increase in cytoplasmic Ca^{2+} levels, primarily due to the influx of extracellular Ca^{2+} [7–9]. We have previously demonstrated that nsPEFs induce PAR formation and necrotic cell death in HeLa S3 cells [17]. To clarify the relationship between extracellular Ca^{2+} and PAR formation, we prepared HeLa S3 cells suspended in either Ca^{2+}-containing or Ca^{2+}-free medium and exposed them to nsPEFs. We performed western blotting using an anti-PAR antibody, and PAR formation could be detected as a smear because PAR is formed on multiple cellular proteins. As shown in Fig. 1, in the presence of extracellular Ca^{2+}, PAR formation was efficiently induced by nsPEFs in accordance with our previous observation. However, in the absence of extracellular Ca^{2+}, PAR formation was nearly absent, indicating a critical requirement of extracellular Ca^{2+} for the nsPEF-induced PAR formation in HeLa S3 cells.

PAR formation is the result, rather than the cause, of nsPEF-induced necrosis

Although PAR formation was suppressed in the absence of extracellular Ca^{2+}, we detected almost equal amounts of PARP-1 between the Ca^{2+}-free and Ca^{2+}-containing samples (Fig. 1) and thus speculated that extracellular Ca^{2+} is required to increase enzymatic activity of PARP-1. To confirm this idea, the experiment was repeated in the presence of PJ34, an inhibitor of PARP-1. As shown in Fig. 2A, PJ34 completely inhibited PAR formation by nsPEFs in the presence of Ca^{2+}, confirming that exposure to nsPEFs led to the increased activity of PARP-1.