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Nanosecond pulsed electric fields induce poly(ADP-ribose) formation and non-apoptotic cell death in HeLa S3 cells

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ABSTRACT

Nanosecond pulsed electric fields (nsPEFs) have recently gained attention as effective cancer therapy owing to their potency for cell death induction. Previous studies have shown that apoptosis is a predominant mode of nsPEF-induced cell death in several cell lines, such as Jurkat cells. In this study, we analyzed molecular mechanisms for cell death induced by nsPEFs. When nsPEFs were applied to Jurkat cells, apoptosis was readily induced. Next, we used HeLa S3 cells and analyzed apoptotic events. Contrary to our expectation, nsPEF-exposed HeLa S3 cells exhibited no molecular signs of apoptosis execution. Instead, nsPEFs induced the formation of poly(ADP-ribose) (PAR), a hallmark of necrosis. PAR formation occurred concurrently with a decrease in cell viability, supporting implications of nsPEF-induced PAR formation for cell death. Necrotic PAR formation is known to be catalyzed by poly(ADP-ribose) polymerase-1 (PARP-1), and PARP-1 in apoptotic cells is inactivated by caspase-mediated proteolysis. Consistently, we observed intact and cleaved forms of PARP-1 in nsPEF-exposed and UV-irradiated cells, respectively. Taken together, nsPEFs induce two distinct modes of cell death in a cell type-specific manner, and HeLa S3 cells show PAR-associated non-apoptotic cell death in response to nsPEFs.

1. Introduction

Ultrashort electric pulses, such as nanosecond pulsed electric fields (nsPEFs), have attracted considerable attention for their unique biological effects. Exposure of cultured cells to nsPEFs elicits varying cellular responses according to nsPEF intensity. Relatively mild exposure activates stress responses [1] and intracellular signal pathways, such as MAPK pathways [2] and AMPK pathway [3]. Conversely, intense nsPEFs efficiently induce cell death *in vitro* [4–6] and tumor regression *in vivo* [7,8], indicating the clinical potential of nsPEFs in cancer therapy.

Cell death can be classified into several distinct forms according to characteristic features, and previous studies have demonstrated that nsPEFs induce apoptosis in several cell lines, including Jurkat cells [4,6]. Apoptosis has been established to proceed via the sequential activation of intracellular events comprising initiation and execution phases [9]. Caspases play critical roles in the progression of apoptosis and are categorized into two groups, initiators and effectors, that are involved in the initiation and execution phases, respectively [9]. Activation of the initiation phase alone is insufficient to confer apoptotic phenotypes, and enzymatic activities of effector caspases are necessary for apoptosis. Caspase 3 is a major effector caspase and is involved in many key events during apoptosis. Effector caspases exist in an inert form in normal cells and are activated by limited proteolysis. Activated effector caspases conduct proteolysis of various cellular proteins, leading to manifestations of apoptosis. Because proteolysis is essentially irreversible, proteolysis-mediated events in apoptosis are regarded as reliable manifestations of apoptosis. Another characteristic of apoptosis execution is bulk degradation of chromosomal DNA into nucleosomal units, a process that is also irreversible and therefore widely used to identify apoptosis [10]. Furthermore, externalization of phosphatidylserine is generally considered another example of an apoptosis marker. Notably, in nsPEF-exposed cells, phosphatidylserine externalization occurs independently of apoptosis, because of the direct effects of nsPEFs on the plasma membrane [11].

In addition to apoptosis, cell death includes multiple forms of non-apoptotic ones, a representative example of which is necrosis [12]. Necrosis is induced by severe disturbances in cellular physiology, such as energy deprivation, and necrotic cells exhibit defects in cellular homeostasis, including ion imbalance and consequent cell swelling [12,13]. Necrosis has long been postulated as a pas-

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

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sive and uncontrolled way of cell death, but it has become evident that elaborate mechanisms underlie some forms of necrosis [14], although their molecular details remain to be fully understood. Furthermore, accumulating evidence has indicated the functional association of necrosis with various physiological and pathological characteristics [12]. In particular, when apoptosis is impeded, necrosis frequently occurs as an alternative means of cell death [12,13].

Whereas apoptosis exhibits many distinct biochemical features, necrosis demonstrates a limited number of molecular signatures, one of which is the persistent presence of protein modification with a polymer of ADP-ribose (poly(ADP-ribose), PAR) [15,16]. Protein modification with PAR is primarily catalyzed by poly (ADP-ribose) polymerase-1 (PARP-1) [16]. In normal cells, PARP-1 plays an important role in DNA repair. PAR is also formed during apoptosis and necrosis in different temporal patterns. In apoptosis, DNA fragmentation elicits DNA damage-induced PARP-1 activation, leading to transient PAR formation. Because DNA repair generally interferes with the execution of apoptosis, PARP-1 is rapidly cleaved by effector caspases to promote apoptosis, and PAR quickly disappears with the progression of apoptosis [17]. In necrosis, the catalytic activity of PARP-1 is persistently elevated, resulting in robust PAR formation that promotes necrotic cell death [15,16]. Thus, the status of PARP-1 reflects the modes of cell death; PARP-1 remains intact in necrosis, whereas the presence of cleaved PARP-1 indicates the execution of apoptosis.

In this study, we analyzed the mode of cell death in Jurkat and HeLa S3 cells exposed to nsPEFs. Although nsPEF exposure induced apoptosis in Jurkat cells, HeLa S3 cells did not exhibit major molecular hallmarks for apoptosis execution after nsPEF exposure. Instead, persistent PAR formation occurred after intense nsPEF exposure. We observed that PARP-1 was cleaved after UV irradiation but remained intact after intense nsPEF exposure. These results demonstrate that distinct mechanisms are involved in nsPEF-induced cell death, and that HeLa S3 cells use non-apoptotic cell death after exposure to nsPEFs.

2. Materials and methods

2.1. Cell culture, nsPEF exposure, and UV irradiation

HeLa S3 cells were cultured in αMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Jurkat cells were maintained in RPMI-1640 supplemented with 10% FBS and penicillin/streptomycin.

For exposure to nsPEFs, a cell suspension (400 μl) was placed in an electroporation cuvette with a pair of 4-mm gapped aluminum electrodes (#5540, MβP, Thermo Fisher Scientific). Shots of nsPEFs were generated using a pulsed power modulator (Supplementary Fig. 1A) and applied to a cell suspension at 1 Hz. A typical example of waveforms under our standard experimental conditions is shown in Supplementary Fig. 1B, and the pulse width at 50% maximum was estimated to be approximately 80 ns. Aliquots of the nsPEF-exposed cell suspension were diluted in prewarmed fresh media and incubated at 37 °C for the periods indicated in the figures. For inhibition of caspase activities, cells were pretreated with 20 μM z-VAD-fmk (Enzo Life Sciences) for 30 min, exposed to nsPEFs, and diluted in media containing the same concentration of z-VAD-fmk. For UV irradiation, HeLa S3 cells were exposed to 312 nm UV light as described previously [18].

2.2. Measurement of cell viability

Cells were exposed to nsPEFs as described above, immediately diluted with fresh media, and incubated at 37 °C for 16 h. Cell viability was measured by the MTT method with a Cell Proliferation Kit I (Roche Applied Science) according to manufacturer's procedures.

2.3. DNA fragmentation assay

Cells were treated with UV irradiation or nsPEF exposure as described above and incubated at 37 °C for the periods indicated. Cells were lysed in a buffer containing 50 mM Tris–Cl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 0.2% SDS, 200 μg/ml proteinase K, and 25 μg/ml RNase A. The lysates were incubated at 37 °C for 16 h. After brief centrifugation, DNA was collected by ethanol precipitation and resolved by agarose gel electrophoresis, followed by ethidium bromide staining.

2.4. Western blot analysis

Cells were pelleted by brief centrifugation and were directly lysed in SDS–PAGE loading buffer that contained 1% SDS. After incubation at 100 °C for 10 min, the cell lysates were briefly sonicated with a sonicator (Model UR-20P, Tomy Seiko). Proteins in the lysates were resolved by SDS–PAGE, electrotransferred to PVDF membrane, and reacted with primary antibodies. A monoclonal antibody against PAR (clone 10H) was obtained from Tulip BioLabs. Antibodies against PARP-1 and caspase 3 were obtained from Cell Signaling Technology. An antibody for β-actin was purchased from Sigma–Aldrich. A monoclonal antibody against Ku80 was a kind gift from Dr. David Chen (University of Texas Southwestern Medical Center). Antigen–antibody complexes were reacted with HRP-conjugated secondary antibodies and detected using the chemiluminescence method as described previously [2].

2.5. In situ detection of cleaved caspase

Cells were fixed in 4% paraformaldehyde at 4 °C for 30 min and stained with an antibody that recognizes the cleaved forms of caspase 3 (Cell Signaling Technology). Detailed procedures are described in Supplementary Information.

3. Results

3.1. Exposure of HeLa S3 cells to nsPEFs caused non-apoptotic cell death

Previous studies have demonstrated that nsPEFs induce apoptosis in several cultured cell lines, one of which is Jurkat cells [4,6]. To determine the conditions of nsPEFs for the induction of cell death, we applied various shot numbers of nsPEFs to Jurkat and HeLa S3 cells and examined their cell viability. As reported previously [5], Jurkat cells were relatively sensitive to nsPEFs, and 20 shots of nsPEFs at 20 kV/cm reduced cell viability to approximately 13% (Fig. 1A). Under the same conditions, we observed DNA ladders in Jurkat cells (Fig. 1B). Although the fluorescence signals of the DNA ladders caused by nsPEF exposure were weaker than those induced by UV irradiation, their presence indicates apoptosis execution in Jurkat cells after nsPEF exposure.

Next, we repeated the experiments using HeLa S3 cells. Compared to Jurkat cells, HeLa S3 cells were relatively resistant to nsPEF exposure; 40 shots of 20 kV/cm nsPEFs were required to reduce cell viability (approximately 18%). Under these nsPEF conditions (40 shots, 20 kV/cm), we detect no DNA ladder on agarose gel electrophoresis (Fig. 1C). Because strong fluorescence of high-molecular-weight DNA interfered with precise inspection of DNA with lower molecular weight, we cut away the portion of agarose gel corresponding to large chromosomal DNA (Fig. 1C, lower panel)

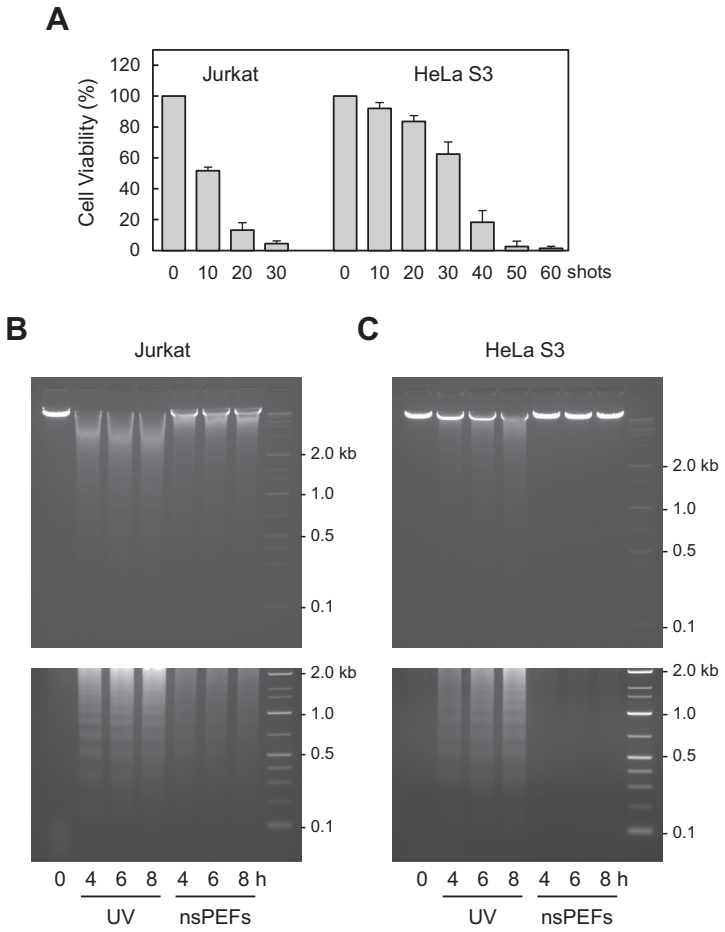

Fig. 1. Reduction in cell viability by nsPEFs is associated with DNA ladder formation in Jurkat cells but not in HeLa S3 cells. (A) Cell viability of Jurkat and HeLa S3 cells at 24 h after nsPEF exposure. Jurkat and HeLa S3 cells were exposed to the indicated shots of nsPEFs at 20 kV/cm, and cell viability at 24 h after nsPEF exposure was measured using the MTT method. Experiments were repeated six times, and the average values with standard deviation are indicated. (B) DNA ladder formation in Jurkat cells. Jurkat cells were treated with UV irradiation at 100 mJ/cm^2 or exposure to 20 shots of nsPEFs at 20 kV/cm. Genomic DNA was isolated from cells after incubation for the indicated times and resolved by agarose gel electrophoresis followed by ethidium bromide staining. The upper panel shows the whole gel, in which strong fluorescence arises from high-molecular-weight DNA. To highlight DNA ladder formation, the portion of agarose gel corresponding to large DNA beyond approximately 2 kb was cut away (lower panel). (C) DNA ladder formation in HeLa S3 cells. HeLa S3 cells were irradiated with 100 mJ/cm^2 UV or exposed to 40 shots of nsPEFs at 20 kV/cm. DNA ladder formation was analyzed as described in (B).

but still found no DNA ladder in nsPEF-exposed HeLa S3 cells. However, DNA ladder was easily detected in UV-irradiated HeLa S3 cells, indicating the capability of those cells to execute apoptosis (Fig. 1C).

Next, we analyzed the status of caspase 3 in HeLa S3 cells after nsPEF exposure and UV irradiation, because cleaved forms of caspase 3 are generally considered to be the most common indicators of apoptosis execution. As shown in Fig. 2A, 20 shots of nsPEFs induced caspase 3 cleavage in Jurkat cells, which is in good agreement with the appearance of the DNA ladder under the same condition. In HeLa S3 cells, caspase 3 cleavage was induced by UV irradiation, but not by nsPEF exposure (Fig. 2B). To confirm the absence of cleaved caspase 3 in nsPEF-exposed HeLa S3 cells, we assessed individual cells by microscopy after immunostaining with an antibody that reacts with cleaved forms of caspase 3. As shown in Fig. 2C, fluorescence of cleaved caspase 3 was evident in UV-irradiated cells but virtually absent in nsPEF-exposed cells. Typical images of microscopy are shown in Supplementary Fig. 2. Taken together with the absence of DNA ladder formation, these observations demonstrate that reduced cell viability after nsPEF exposure is not associated with apoptosis execution in HeLa S3 cells.

3.2. Intense nsPEFs induced PAR formation in HeLa S3 cells

The absence of major molecular manifestations of apoptosis execution in nsPEF-exposed cells prompted us to search for intracellular events related to non-apoptotic cell death. Necrosis is a major type of non-apoptotic cell death and shows a relatively limited number of molecular signatures, one of which is PAR formation (Fig. 3A) [15,16]. Because varying numbers of ADP-ribose are conjugated to multiple proteins, PAR formation can be detected as a high-molecular-weight smear in western blots. As shown in Fig. 3B, we observed smears of PAR signals in western blotting of nsPEF-exposed HeLa S3 cells. PAR formation was late-onset and increased gradually after nsPEF exposure (Fig. 3B). We reprobed the western blot membrane with an anti-PARP-1 antibody and observed constant amounts of PARP-1 among the western blot samples, supporting the notion that the enzymatic activity, but not the amount, of PARP-1 was elevated after nsPEF exposure. Next, we applied different shot numbers of nsPEFs and performed western blotting of PAR. As shown in Fig. 3C, 40 shots of nsPEFs yielded remarkable PAR formation. Notably, in the MTT assays shown in Fig. 1A, 40 shots of nsPEFs achieved marked reduction in cell viability, suggesting the correlation between PAR formation and reduced cell viability in

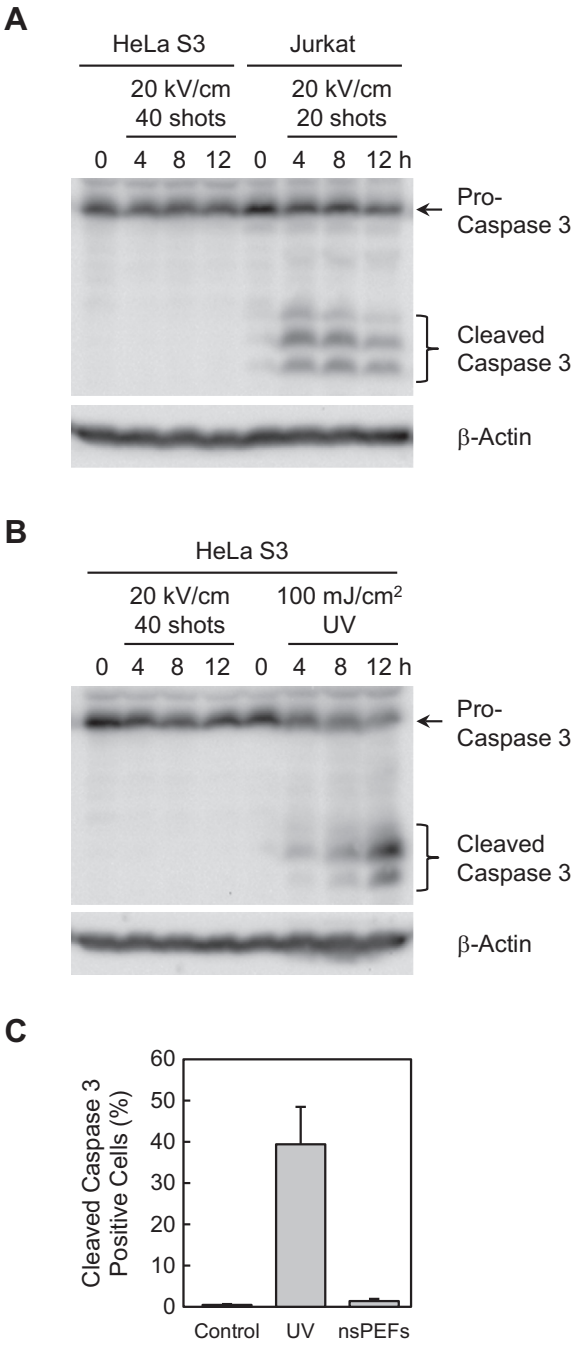

Fig. 2. Proteolytic activation of caspase 3 does not occur in HeLa S3 cells exposed to nsPEFs. (A) Western blot analysis of caspase 3 cleavage in Jurkat and HeLa S3 cells. Jurkat and HeLa S3 cells were exposed to nsPEFs and incubated for the indicated times. Western blotting was performed using an antibody that reacts with both inactive and cleaved forms of caspase 3. As a loading control, β-actin was detected. (B) Western blot analysis of caspase 3 in HeLa S3 cells treated with either nsPEF exposure or UV irradiation. HeLa S3 cells were treated with UV irradiation or nsPEFs, and western blotting was performed as described in (A). (C) Quantification of cleaved caspase 3-positive cells. HeLa S3 cells were treated with UV irradiation (100 mJ/cm^2) or nsPEF exposure (40 shots at 20 kV/cm) and incubated for 10 h. Cells were stained with an antibody that recognizes cleaved forms of caspase 3. Cells with cleaved caspase 3 were scored using fluorescence microscopy. The total cell number was obtained from DAPI-staining, and at least 300 cells were counted in each experiment. Average values for 6 independent experiments and standard deviations are shown in a graph.

nsPEF-exposed cells. Collectively, these observations demonstrate that intense nsPEFs elicit non-apoptotic cell death that is associated with PAR formation in HeLa S3 cells.

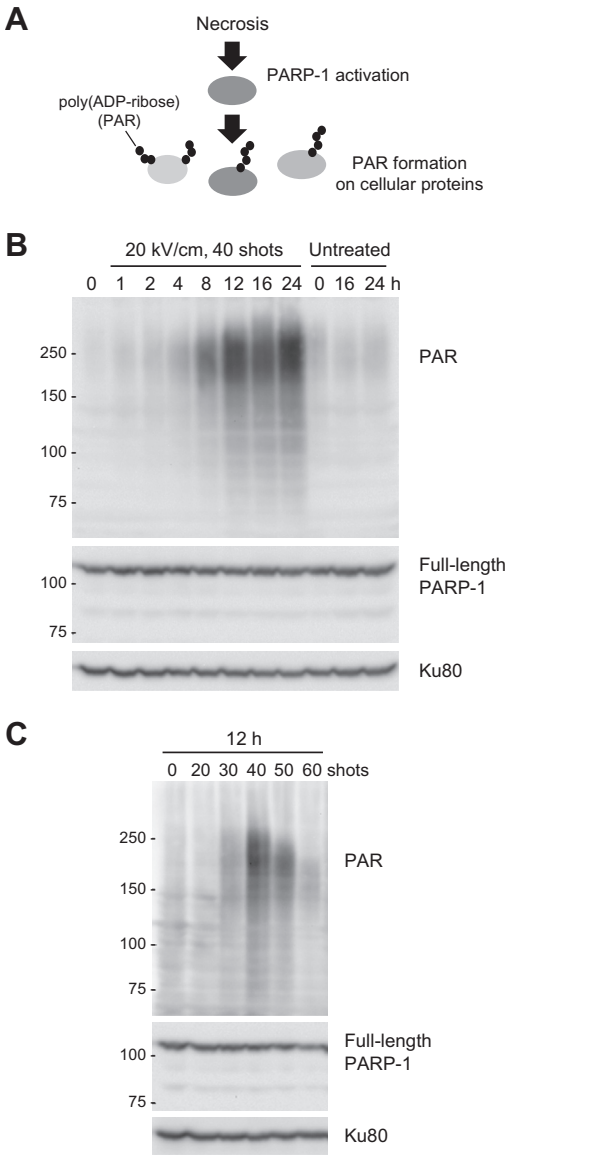

Fig. 3. Exposure to intense nsPEFs elicits PAR formation. (A) Schematic representation of PAR formation in necrosis. In necrosis, PARP-1 activity is dramatically elevated, and PAR is formed on multiple cellular proteins. (B) Time-course analysis of PAR formation after nsPEF exposure. HeLa S3 cells were exposed to 40 shots of nsPEFs at 20 kV/cm. The cells were collected at the indicated times and used for western blot analysis of PAR. PARP-1 and Ku80 are shown as loading controls. (C) Exposure to intense nsPEFs results in PAR formation. HeLa S3 cells were exposed to the indicated shot numbers of nsPEFs at 20 kV/cm and incubated for at 37 °C for 12 h. Western blot analysis was performed as described in (B).

3.3. UV and nsPEFs activated distinct mechanisms for cell death in HeLa S3 cells

In apoptosis, PARP-1 is inactivated through cleavage by effector caspases (Fig. 4A) [17]. Caspase 3 is also processed via proteolysis, resulting in activation [9]. In necrosis, PARP-1 and caspase 3 remain intact, due to the lack of the activation of effector caspases (Fig. 4A). The pan-caspase inhibitor z-VAD-fmk can suppress apoptotic proteolysis. To confirm the modes of cell death induced by UV and nsPEFs, we analyzed the status of PARP-1 and caspase 3 in the presence and absence of z-VAD-fmk. First, we performed western blot analysis of UV-irradiated HeLa S3 cells. As shown in Fig. 4B, we observed that proteolytic processing of both PARP-1 and caspase 3 were partially suppressed by z-VAD-fmk. Next, we carried out western blot analysis of nsPEF-exposed cells and detected

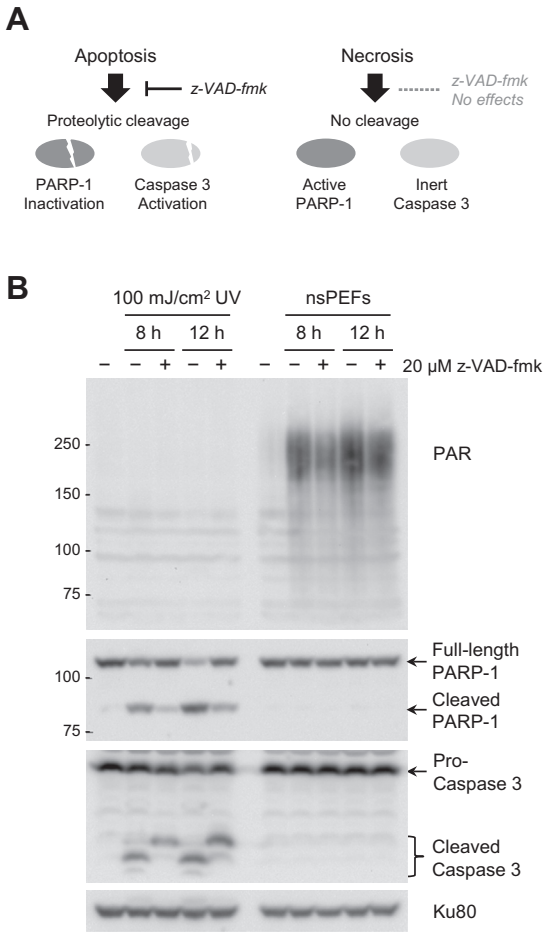

Fig. 4. UV and nsPEFs induce different pathways for cell death in HeLa S3 cells. (A) Schematic representation of the PARP-1 and caspase 3 status. In apoptosis, caspase-mediated proteolysis activates caspase 3 and inactivates PARP-1, which can be suppressed by z-VAD-fmk, a pan-caspase inhibitor. In necrosis, both PARP-1 and caspase 3 remain intact. The enzymatic activity of PARP-1 is elevated, whereas caspase 3 remains inert. (B) Western blot analyses of PAR, PARP-1, and caspase 3 in the presence and absence of z-VAD-fmk. HeLa S3 cells were treated with either UV irradiation or nsPEF exposure and incubated at 37 °C for the indicated times in the presence or absence of 20 μM z-VAD-fmk. Western blot analyses of PAR, PARP-1, and caspase 3 were performed. Ku80 is shown as a loading control.

intact forms of PARP-1 and caspase 3, along with elevated PAR formation (Fig. 4B). These observations support the idea that UV and nsPEFs induce apoptotic and necrotic cell death, respectively, in HeLa S3 cells.

4. Discussion

Previous studies have reported nsPEF-induced apoptosis in several cell lines [4,6]. Accordingly, we observed the major biochemical manifestations of apoptosis execution in Jurkat cells after nsPEF exposure. Conversely, HeLa S3 cells exposed to nsPEFs exhibited no major signs of apoptosis execution. Although apoptotic cells do not necessarily exhibit a whole set of apoptotic features, a total lack of biochemical indications of apoptosis execution suggests non-apoptotic cell death in nsPEF-exposed HeLa S3 cells. Considering the presence of elevated PAR formation, nsPEF exposure appears to lead to necrosis in HeLa S3 cells.

This study raises several questions for further investigations. First, a precise classification of nsPEF-induced non-apoptotic cell death is needed, although the presence of PAR suggests necrosis. Whereas apoptosis is well defined by many distinct molecular events, a limited number of molecular signatures have been documented in necrosis thus far. Historically, necrosis has been judged by the absence of major apoptosis markers and by failure of normal cellular physiology, such as decreased energy production [12]. Recent studies have demonstrated that regulated mechanisms are involved in the execution of some forms of necrosis [14], although the molecular mechanisms for such necrosis remain to be fully elucidated. Detailed characterization of nsPEF-induced non-apoptotic cell death with identification of key molecular events will help us understand the modes of nsPEF-induced cell death more precisely.

The second question is why apoptosis is not induced after nsPEF exposure, even though HeLa S3 cells are capable of executing apoptosis after UV irradiation. Apoptosis relies on the ordered activation of initiation and execution phases, and the activation of the initiation phase alone is generally insufficient to produce the apoptotic phenotype [9]. Many studies have demonstrated that progression to the execution phase can be halted under certain circumstances. For example, ATP depletion prevents the formation of functional apoptosome and consequently restricts the activation of effector caspases in the intrinsic apoptosis pathway [13,19]. When such restriction occurs, necrosis frequently occurs as an alternative mechanism for cell death [12]. Thus, a possible explanation is that intense nsPEFs have detrimental effects on the execution of apoptosis, leading to a shift from apoptosis to necrosis, but additional detailed analysis is required to test this idea.

The third question is which cellular effects of nsPEFs trigger necrosis. Previous studies have indicated that nsPEFs act at multiple sites in cells and induce distinctive effects, most of which appear to have positive effects on necrosis induction. One of the major sites of nsPEF action is the plasma membrane, which is directly impacted by nsPEFs to generate small pores that allow influx of extracellular calcium and other ions [11,20]. Intriguingly, necrosis is markedly facilitated by membrane damage, calcium overload, and ion imbalance [12,21]. Furthermore, a recent study has demonstrated that nsPEF exposure produces reactive oxygen species [22], which also facilitate necrosis. In addition, in several cell lines, nsPEFs are reported to elicit Bax activation and mitochondrial dysfunction, both of which are well-documented in apoptosis and have additional positive effects on necrosis [6,23]. Thus, the cumulative burden of nsPEF effects may trigger necrosis when apoptosis execution is restricted.

The next question involves cell-type specificity and prevalence of PAR-associated non-apoptotic cells among various human cell lines. This study focused on the characterization of biological effects of nsPEFs in Jurkat and HeLa S3 cells. Additional experiments (data not shown) revealed PAR formation and a lack of major biochemical manifestations of apoptosis in several cell lines. These results will be presented in a future study. Thus, we think that nsPEF-induced non-apoptotic cell death with PAR formation is not an exceptional observation using a particular cell line. Future efforts to survey the modes of cell death induced by nsPEFs among various cell lines will provide important information on the cell specificity of nsPEF effects.

Finally, our findings in this study provide new insights into the clinical application of nsPEFs in cancer therapy. Cancer is intrinsically resistant to apoptosis, because acquisition of apoptosis resistance is essential for tumorigenesis [12]. Furthermore, defects in apoptosis pathways confer resistance to many anti-tumor agents, the actions of which rely on intact apoptosis mechanisms. Although defective apoptosis is intimately associated with resistance to anti-tumor agents, cell death can still be induced in many cancer cells via non-apoptotic mechanisms, because mechanisms for apoptotic and non-apoptotic cell death are distinct from each other [12,24]. Recently, considerable attention has been given to

the clinical significance of non-apoptotic cell death, because it is the predominant mode of cell death in several clinical situations. One prominent example is photodynamic therapy, which often leads to necrosis rather than apoptosis [25]. Previous studies of nsPEF-induced apoptosis have clearly demonstrated the clinical potential of nsPEFs in cancer therapy. In addition to apoptosis induction, observations of non-apoptotic cell death after nsPEF exposure provide additional rationale for the therapeutic application of nsPEFs. A better understanding of the molecular mechanisms underlying nsPEF-induced non-apoptotic cell death will underscore the potential of nsPEFs and accelerate their application in cancer therapy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.083.

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