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Non-Ionizing Radiation with Nanosecond Pulsed Electric Fields as a Cancer Treatment: *In Vitro* Studies

Stephen J Beebe, Wentia E. Ford, Wei Ren, Xinhua Chen, and Karl H. Schoenbach

Abstract - Cancer continues to be a major risk to the health and well being among populations around the world. A new method using ion-ionizing radiation with nanosecond pulsed electric fields (nsPEFs) provides a novel means to treat cancer at local sites. NsPEFs promote cell death in several cell types and here we investigated mechanisms for cell death induction. In murine B16f10 melanoma, murine E4 squamous carcinoma, murine Hep1-6 and human HepG2 hepatocellular carcinoma, nsPEFs induced cell death in 90-95% of cells. Cell death coincides with decreases in the mitochondria membrane potential, increases in YO-PRO-1 uptake and active caspases in the presence or absence of cytochrome c release. The results indicate that nsPEFs induced cell death by multiple apoptosis mechanisms that involve mitochondrial responses, but not necessarily through cytochrome c release. Further, these *in vitro* studies suggest a potential to induce cell death that bypasses cancer mechanisms that evade apoptosis.

INTRODUCTION

The National Cancer Act began a war on cancer in 1971. The biology of cancer has proved to be a more formidable enemy than anticipated and 38 years later the fight to find effective treatments for cancer continues. It has become clear that there are no "silver bullets" to conquer these diseases. Cancer is not a single disease and what is effective for one may not be for another. Now nearly all cancers are treated with combinations of agents that consist of ionizing radiation and substantial numbers of chemotherapeutic agents that include antineoplastic drugs, immunomodulatory agents, targeted therapy (monoclonal antibodies, signal transduction inhibitors), hormone therapy and/or biologic response modifiers. Cancer causing mutations occur at multiple sites in an atlas of cancer pathways, which when illustrated resembles an electronic circuit board. Oncologists are clearly looking for new therapies more effective and less toxic. Several newer treatment strategies use electroporation as therapeutics. Electro-gene transfer uses electroporation to deliver genes that signal the immune system against the tumors [1]; electrochemotherapy uses electroporation to deliver a chemotherapeutic agent [2]-[3] and irreversible electroporation uses electroporation to induce necrosis [4] Other methods use temperature such as cryo-therapy, which induces necrosis by freezing and radiofrequency ablation, which induces thermal necrosis [5].

A new emerging therapy that has not reached the clinic is the use of non-ionizing radiation from nanosecond pulsed electric fields (nsPEFs). This approach does not use drugs or thermal mechanisms, but extends the use of electric fields beyond electroporation in that it decreases the pulse duration into the nanosecond range and increases the electric field into tens and hundreds of kilovolts / centimeter. Use of non-ionizing radiation with nsPEFs is emerging as a means for apoptosis induction in tumor cells and tissues [6]-[12] as well as a means to induce vascular instability and potential infarctive tumor death *in vivo* [13]-[14]. Unlike conventional electroporation, nsPEFs are high in power (megawatts instead of watts) and non-thermal, low energy density (millijoules/cc instead of joules/cc). Furthermore, experimental evidence [15]-[18] and modeling evidence [19]-[23] suggest that as the pulse duration decreases, effects on intracellular membranes become an important factor in the mechanisms of action of nsPEFs in cells and tissues. Unlike conventional electroporation, which forms relatively large pores in the plasma membrane only, nsPEFs form nanopores in all cell membranes including intracellular organelles and limits distribution of molecules larger than a about a nanometer across these membranes [19]-[23]. These nsPEF-induced effects have been called supra-electroporation by Weaver and colleagues and they hypothesized that these nanopores play important roles in how cells respond to nsPEFs [20]. It remains to be determined how these nanopores influence cell responses.

NsPEFs act to trigger cell functions, including activating signals that are below and above the threshold for cell death, although there are cell type specific differences. For example, using non-lethal conditions, nsPEFs induce calcium mobilization [6], [9]-[11], [16], activate human platelets [24] activate L-type calcium channels [25] and induce phagocytosis in cells that do not exhibit active caspases [17]. In contrast, when the electric field is sufficiently intense, all cells tested thus far exhibit apoptosis-like signs [6]-[11]. Mouse B16f10 tumors exhibit caspase activation [Chen and Beebe, unpublished data] and furthermore, these tumors are eliminated in 1-2 weeks [13]-[14].

In order to determine if nsPEFs induced programmed cell death by apoptosis, we have used several markers that had been shown to be harbingers of apoptosis including plasma

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Stephen J. Beebe was previously at Eastern Virginia Medical School where some of this work was done and is now with the Frank Reidy Research Center for Bioelectrics, Old Dominion University (phone: 757-683-2405, Fax: 757314-2397; e-mail: sbeebe@odu.edu). Wentia Ford is a post-doctoral fellow at Old Dominion University; Wei Ren is a PhD graduate student in the Frank Reidy Research Center for Bioelectrics at Old Dominion University; Xinhua Chen is a post-doctoral fellow in the Frank Reidy Research Center for Bioelectrics at Old Dominion University; Karl H. Schoenbach is a professor in the Frank Reidy Research Center for Bioelectrics at Old Dominion University

membrane integrity, cell size changes (forward light scatter), caspase activation, phosphatidylserine externalization, cytochrome c release [8]-[10], [12], and changes in the mitochondria membrane potential [this report]. While nsPEFs induce many of these changes, it is highly likely that apoptosis may function in parallel with or cross talk with other cell survival and death mechanisms. NsPEFs represent an intense stimulus with characteristics that were not present during evolution, suggesting that cells must use mechanisms that evolved for other stimuli that affect cell fate. In these studies we show evidence that nsPEFs can induce cell death in tumor cells *in vitro*, thus bypassing apoptosis evasion mechanisms and providing a potential for a new, non-toxic, safe and inexpensive means to eliminate tumors at local sites. Here we show *in vitro* evidence that support use of nsPEFs as a new cancer therapy.

Materials and Methods:

Cell culture and treatment with nsPEFs: All cell lines were obtained from ATTC and cultured in log phase growth as suggested by the supplier. Cells were exposed to one or ten 60ns or 300ns pulses at various electric fields in cuvettes with a 0.1 cm gap at 7.7 x 10^6 cells / ml (1 x 10^6 cells / 130µl.

Flow cytometry: Fifteen thousand cells were acquired by Becton-Dickinson flow cytometer. Data was analyzed by Cell Quest Pro software.

Measurement of pan caspase activity: Active caspase was determined with FITC-VAD-fmk (20µM) (Promega; Madison, MI). Cells were treated and incubated for 10-20 minute prior to analysis by flow cytometry.

Measurement of caspase 3 activity: Hep1-6 and HepG2 were treated as indicated in Figure 3 and cultured for 1 hour with FAM-DEVD-FMK, selective for caspase 3 (FLICA™ ,Immunochemistry Techonologies, LLC) before analysis by flow cytometry. Results are means ± SE (n=3).

Evaluation of membrane permeability, YO-PRO-1, and Annexin-V-FITC binding: B16f10 melanoma cells were exposed to nsPEF conditions in the presence and absence of 1:5 propidium iodide/RNase A (Invitrogen; Oregon), 0.2µM YO-PRO-1 (Invitrogen; Oregon), Annexin-V-FITC (Alexis Biochemical; San Diego, CA) according to the supplier's recommendation. All analyses were by flow cytometry.

Measurement of cytochrome c release by flow cytometry: Two different procedures were used. Cells were exposed to nsPEFs, incubated with monoclonal antibodies for cytochrome c (BD Pharmingen; San Jose, CA) and then incubated with Alexa Fluor 488 goat anti-mouse antibody (Invitrogen; Oregon) and analyzed by flow cytometry. A second cytochrome c procedure used the InnoCyte cytochrome c release assay as recommended by the suppliers (Calbiochem). This procedure allows cytochrome c to diffuse out of the cell before fixing, resulting in decreased fluorescence with increased cytochrome c release when analyzed by flow cytometry.

Assessment of changes in Mitochondrial Membrane Potential (ΔΨm): Cells were labeled for 5-10 minutes at 37°C with 2µM JC-1 (Molecular Probes, Eugene, Oregon) or 200nM TMRE (Immunochemistry Technologies LLC, Bloomington, MN). The emission of JC-1 changes from red to green when ΔΨm decreases. For TMRE red emissions decrease when ΔΨm decreases. Cells were washed and analyzed by flow cytometry.

Statistical analysis: Statistical analysis was performed using SigmaPlot software. Nominal data were compared using two-way ANOVA followed by Bonferroni t-test. A p value of <0.05 was considered significant.

Results

In early studies, we investigated the correlation of apoptosis markers using flow cytometry in 3T3-L1 mouse embryonic fibroblasts, human Jurkat cells and human HL-60 cells [6]-[10], [15], [16]. While these cell models did not represent *in vivo* models, they provided cell types that were well characterized through a wide range of studies. We found that the presence of active caspases correlated with decrease in size (forward light scatter), the presence ot PS externalization and the absence of PI uptake, especially at early times post pulse, lower pulse durations and electric fields. These results clearly demonstrate four coincident apoptosis markers in the major population (85-95%) of cells. We also demonstrate in Jurkat and HL-60 cells that cytochrome c release occurred in the in the same time frame (30-60minutes) for the appearance of active caspases [8].

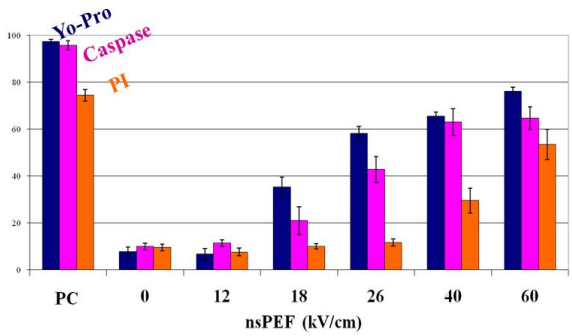

Fig. 1. B16f10 cells were treated with ten 300ns pulses at the indicated electric field and assayed 1 hour post pulses as described in Materials and Methods. PC: positive control: 20% ethanol. The results indicate an electric field-dependent increase in all markers with a higher electric field required for PI entry.

In more recent studies, we have used B16f10 cells an *in vivo* model with nsPEF studies [14], [15] and here investigate *in vitro* characteristics of nsPEFs effects on apoptosis markers. In B16f10 cells the presence of active caspase, an apoptosis hallmark, Yo-PRO-1 uptake, which has been reported to identify apoptotic cells, and propidium iodide (PI) uptake as a measure of membrane integrity were investigated (Figure 1). One hour after the indicated nsPEF conditions, at 18 and 26kV/cm active caspase and Yo-PRO-1 positive cells increase from 10% in the control to 40-50% while PI uptake is not present. As the electric fields increase to 40 and 60kV/cm, active caspase and Yo-PRO-1 positive cells continue to increase as PI uptake becomes evident. In these experiments PI was present during the pulse and the 1 hour incubation. When PI was added at various times after the pulse, uptake did not occur until 15-20 minutes after the pulse and only occurred within the first hour for pulse above 40kV/cm. It is likely that the delayed PI uptake is related to

apoptosis progression as the membrane fails as a post-apoptosis *in vitro* necrosis. Interestingly, in contrast to ethanol, using two different assays, B16f10 cells did not significantly release cytochrome c. Only 15-20% of cells showed cytochrome c release and this did not occur in an electric field dependent manner. Nevertheless, ten 300ns pulses at 60kV/cm killed 90-95% of cells. These results suggest that nsPEFs bypassed apoptosis evasion mechanisms in the cytochrome c pathway in B16f10, yet activated caspases by other mechanisms that remain to be define. Also of interest are the B16f10 mechanisms that prevent cytochrome c release in response to nsPEFs.

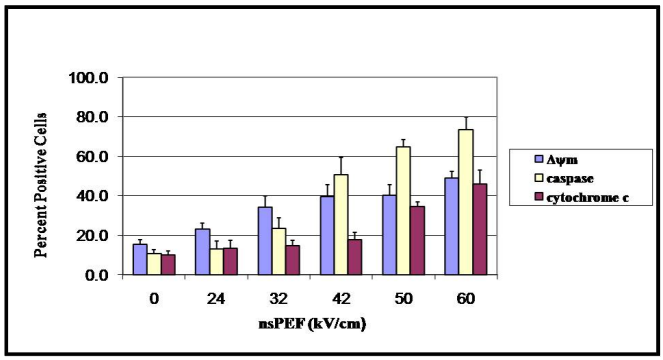

Fig. 2. E4 cells were treated with ten 300ns pulses at various electric fields and analyzed as described in Material and Methods. The results indicate an electric field-dependent increase in all markers with the release of cytochrome c requiring a higher electric field than that required for decrease Δψm and activation of caspases. The Δψm is the most sensitive marker.

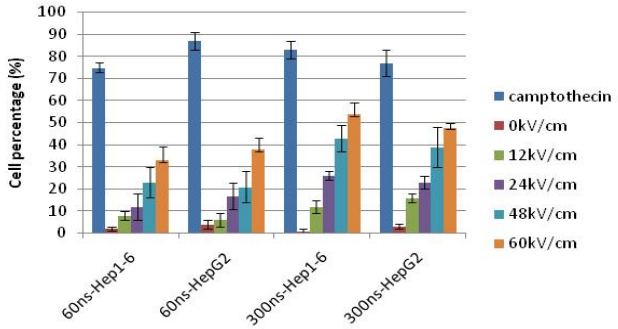

Fig. 3. Cells were exposed to ten 300ns pulses at various electric fields and analyzed for active caspase 3/7 by flow cytometry as described in Material and Methods. Cells were pulsed 10 times and analyzed 3 hours post pulse. The Camptothecin (5 g/ml/) treatment was 3 hours. The results indicate an electric field-dependent increase in the percentage of cells that exhibit active caspase-3/7

We have investigated another skin cancer model, E4 squamous cell carcinoma. In Figure 2 we analyzed the mitochondria membrane potential (Δψm), the presence of active caspase and cytochrome c release one hour after treatment with ten 300ns pulses at various electric fields up to 60kV/cm. The most sensitive indicator to electric field (Figure 2) and time (data not shown) was the decrease in the Δψm. At 60kV/cm Δψm can be observed in 5 minutes using two different methods (see materials and methods). Within the first hour active caspases can be seen above 32kV/cm, but cytochrome c release was only observed at 50kV/cm. Thus at least some of caspase activation is independent of the presence of cytochrome c in the cytosol. Likewise at 50 and 60kV/cm there are greater numbers of cells with active caspases than with cytochrome c released. These results suggest that caspase activation can be independent of cytochrome c, while at the same time mitochondria functions have been altered. Using ten 300ns pulses there is an electric field dependent decrease in survival, such that with 60kV/cm, 90-95% of cells die.

In another series of experiments, we investigated the potential for nsPEFs to be effective against hepatic cancer. We used mouse Hep1-6 and human HepG2 hepatocellular carcinoma and investigated caspase-3/7 activation with ten pulses at 60ns and 300ns at various electric fields (Figure 3). Camptothecin served as the positive control. Both pulse durations at > 24kV/cm significantly activated caspase-3 with as much as 50-60% of cells exhibiting active caspase within the first hour after treatment. In cell survival studies, ten 60ns and 60kV/cm killed only about 60% of the cells. However, in common with survival studies in B16f10 and E4, with ten 300ns pulses there is an electric field dependent decrease in survival, such that with 60kV/cm, 90-95% of cells die.

Discussion

Although the non-ionizing nsPEF pulses exhibit extremely high in electric fields, the pulse durations are short enough that significant energy is not imparted to cells or tissues and thus the pulses are non-thermal. In the studies presented here, a decrease in mitochondrial membrane potential (Δψm) is observed within minutes of pulse applications. Decreases in plasma membrane resistance and loss of the plasma membrane potential were also observed within minutes using patch clamp techniques [26]. It is likely that both of these membranes are near immediate responders, although real-time measurements are compromised by pulse effects on plasma membrane patch stability and the Δψm dye response with JC-1. NsPEFs also significantly "rearrange" the actin cytoskeleton [12], possibly damage DNA [14] and promote cytochrome c release, in some cases as indicated here.

The presence of active caspases is the most telling apoptosis marker since it best defines the processes that involve cell disassembly and this is coincident with YO-PRO-1 uptake, which is reported to only cross the membrane of apoptotic cells [27]. Although Figure 1 shows uptake of PI, this did not occur until 15-20 minutes post pulses (data not shown), suggesting that it was caused by biological responses rather than direct effects on the plasma membrane.

Another hallmark of apoptosis is the release of cytochrome c from the mitochondria into the cytoplasm. This is a major pathway in cells that sustain damage or undergo aging and often exhibits mutations that cause cancer. Many cancer treatments target sites in this pathway and many cancer resistances and recurrences appear through blockade of this pathway. In B16f10 cell that exhibit active caspases, cytochrome c release was not a major response to any nsPEF conditions tested. Similar results were observed in E4 cells albeit at low nsPEFs conditions. This demonstrates that nsPEFs can activate caspases under

conditions of cytochrome c blockade, suggesting that they could bypass cancer causing mutations that block this commonly used pathway. These results also indicate that nsPEFs can utilize multiple caspase activation mechanisms in apoptosis pathways that do and do not require cytochrome c release.

These results in B16f10 cells raise several interesting issues. While cytochrome c release and $\Delta\psi m$ are not necessarily linked, it is more common to observe cytochrome c release without a decrease in $\Delta\psi m$ as opposed to opposite responses in B16f10 cells. Many melanomas exhibit elevated levels of Bcl-2, which prevent cytochrome c release, but the mutations in B16f10 are not known. It is possible that nsPEFs do not sufficiently increase oxidants that are required for cytochrome c release or that these cells have high antioxidant levels such as glutathione or Bcl-2. Roles for the oxidation reduction potential and the levels of antioxidants in these cells require further investigation.

Conclusions:

These studies demonstrate that nsPEFs can induce cell responses that indicate apoptosis induction. In studies with B16f10 tumors in vivo, it is clear that nsPEFs can eliminate tumors in mice that suggest a cure at the primary injection site [13]-[14] Preliminary studies indicate similar results in ectopic Hep1-6 hepatocellular carcinoma in mice.

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