Nanoelectroablation for human carcinoma therapy

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ABSTRACT

We have developed a low energy direct current pulsed electric field therapy for tissue ablation. This therapy applies 100 ns long electric pulses 30 kV/cm in amplitude using a contact electrode and triggers apoptosis in the treated tissue. Here we review the progress that has been made in understanding the mechanisms and targets of nanosecond pulsed electric fields (nsPEF) when applied to cells and tissues. This work began in 2001 in the laboratory of Karl Schoenbach who collaborated with biologists Stephen Beebe and Stephen Buescher to demonstrate the permeabilization of intracellular organelles. Since then over 100 papers have been published studying the cellular responses to nsPEF. We discuss these targets and cellular responses and introduce some new results from our group using nanoelectroablation to treat human pancreatic carcinoma in a murine xenograft model system. We have determined that 500 pulses 100 ns long and 30 kV/cm in amplitude are sufficient to ablate human pancreatic carcinomas growing in immunosuppressed mice and these ablated tumors do not recur for at least 300 days. We have also determined that the reactive oxygen species generation that is triggered within a minute after nsPEF treatment is Ca^{2+}-dependent. In order bring this therapy into the clinic for the treatment of human tumors we are developing both a pulse generator as well as delivery electrodes to target the tumors to be treated. We describe the NanoBlate® Model NB-1 100 ns pulse generator and the first human clinical trial data using nanoelectroablation to ablate basal cell carcinomas without scarring.

Keywords: pulsed electric field, basal cell carcinoma, apoptosis, nanoelectroablation, soft tissue ablation, allograft, xenograft

1. INTRODUCTION

Pulsed power technology has been applied to living cells since the 1950's and was first used to generate transient increases in the permeability of the plasma membrane that was called "electroporation". When fields on the order of 1 volt per membrane are applied, they cause a breakdown in the lipid bilayer of the cell's plasma membrane[1]. This results in a water-filled pathway across the lipid membrane that allows ions and other small molecules to cross the normally impermeable lipid bilayer. These first studies used pulsed fields in the microsecond and millisecond domains and field strengths on the order of 1 kV/cm. The pores that formed were transient and reversible unless the field strength was increased to 2-3 kV/cm in which case irreversible pores were formed[2] that killed cells by necrosis.

In 2001, much shorter pulses in the nanosecond domain with field strengths of 10-100 kV/cm were applied to cells for the first time[3]. These shorter pulses also introduce transient, water-filled defects across the plasma membrane but only molecules smaller than 1 nm in diameter could cross the membrane through these transient pathways[4, 5]. These nanosecond pulsed electric fields (nsPEF) were found to have profound effects on treated cells that I will discuss below. The most important characteristic of nsPEF is its ability to penetrate into the cell cytoplasm to permeabilize organelle membranes as well as the plasma membrane[3, 6]. Over the past 12 years, more than 100 studies have been published applying this nsPEF technology to various cell and tissue types.

2. TARGETS OF NSPEF

All cells are bounded by an outer plasma membrane normally composed of a lipid bilayer with associated glycoproteins. This membrane exhibits very low conductivity as compared to the cytoplasm and extracellular fluid so the cell can be modeled as a conductor surrounded by an insulating layer. This is generally true also for organelles within the cytoplasm of cells. When an electric field is imposed across the cell, ions in the cytoplasm will respond to the imposed electric field by rapidly moving in the field direction to charge the capacitance of the membrane until they experience no further force. By definition this will only occur when their redistribution establishes an equal and opposite field so that the net field in the cell interior is zero. However, this redistribution takes a certain amount of time that is characterized by the charging time constant of the plasma membrane and is typically in the 0.1-1μs range. For pulsed fields shorter than this,

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the charges will not have sufficient time to redistribute and counteract the imposed field and it will penetrate into the cell and charge every organelle membrane for a duration that is dependent on both the charging time of the plasma membrane and the organelle membrane. This has been very thoroughly presented in a review chapter by Schoenbach[7]. This ability to penetrate into the cytoplasm allows nsPEF to permeabilize the organelle membranes as well as the plasma membrane. That is a major difference between nsPEF and the original electroporation protocols using longer pulses.

Once the field penetrates into the cytoplasm, the ability to permeabilize the much smaller organelles will require a much higher field strength. Organelle sizes range from the large nucleus (2-6 μm) to much smaller endoplasmic reticulum tubular network and mitochondria in the 0.3-1 μm range. Thus in order to achieve the permeabilizing field of 1 volt across these much smaller organelles, fields of 1 volt over 0.3-1 μm (10-33 kV/cm) are required. These field strengths are an order of magnitude higher than those used for the original electroporation work using microsecond and millisecond pulse lengths.

An important consideration when applying such large electric fields is the energy delivered to the cell. Joule heating is calculated by a simple relationship of current times voltage times time. Thus the original electroporation pulses of 1 kV/cm 100 μs long into a load of 50 ohms would deliver 2 joule of energy into a cubic centimeter of volume. Assuming that the treated tissue has a specific heat similar to that of water (4.1 J/gm-^{o}C), this would increase the temperature of the electroporated tissue by 0.5 ^{o}C, which is an insignificant amount. However, applying the much higher 33 kV/cm required to permeabilize organelles would generate 2178 J, which would vaporize the tissue. This illustrates one important advantage of using the much shorter pulses in the nanosecond domain. Since these pulses are 1000-fold shorter, the energy delivered is also 1000-fold less so that the 33 kV/cm pulse amplitude can be applied and generates only 2.17 J which will increase the tissue temperature by only 0.5 ^{o}C. Therefore nsPEF is able to both penetrate into the cytoplasm and also apply a field strength large enough to influence organelle permeability. These two critical features of nsPEF make it a very powerful tool as will be illustrated further below. This ability to transiently permeabilize intracellular organelles was originally referred to as “electromanipulation” by Schoenbach[8] and subsequently named “supra-electroporation” by Weaver[9, 10]. Recent advances in molecular dynamics have allowed for the modeling of pore formation by nsPEF and these simulations suggest that pores form as quickly as 3 ns after the field is applied[11].

3. OBSERVED CELLULAR RESPONSES TO NSPEF

Temporary immobilization of nuisance species

One of the very first publications applying nsPEF involved biofouling and removal of bacteria. Temporary immobilization of aquatic nuisance species through application of short electric pulses was demonstrated as a method to prevent biofouling in cooling water systems where untreated lake, river, or seawater is used[12]. That same year Schoenbach, Dobbs and Beebe showed 'cold' bacterial contamination of liquid food and drinking water was possible[13].

Apoptosis induction and tumor growth inhibition

The next application of nsPEF came from the collaboration of Schoenbach’s group with Beebe’s group in which they first demonstrated that tumor growth could be inhibited by nsPEF application[14]. In that now classic paper they also demonstrated phosphatidylserine (PS) externalization as a marker of apoptosis. In the 10 years following that paper, Beebe has published more than 25 more papers characterizing the apoptotic response. This includes DNA fragmentation[14], the release of cytochrome C[15] and activation of caspase activity[16-21] in addition to the PS externalization. His group has made the most progress in understanding the steps in the apoptotic pathway triggered by nsPEF. They have shown that nsPEF does not activate extrinsic apoptotic pathways using cells deficient in FADD or caspase-8[22]. However nsPEF does trigger intrinsic apoptosis pathways by releasing cytochrome c from the mitochondria through an APAF-1- and caspase-dependent pathway.

We have been working to develop a therapy to ablate tumors implanted in mice with a single treatment[23-26]. We started with the B16 murine melanoma allograft model system in which murine melanoma cells injected beneath the skin grow into a melanoma that can be treated with nsPEF. We found that a single treatment of 2000 pulses 100 ns in duration and 30 kV/cm in amplitude was sufficient to ablate these tumors[23-26]. We wanted to study the effectiveness of nsPEF to treat more naturally occurring melanomas and this lead us to collaborate with Ed DeFabo who had developed a UV-induced melanoma model using C57/BL6 HGF/SF mice. We successfully ablated all 27 melanomas treated in 14 of these mice using 2000 pulses 100 ns long and 30 kV/cm in amplitude[27]. We coined this treatment, “non-thermal nanoelectroablation”. All nanoelectroablated melanomas gradually disappeared over a period of 12-29 days. Pyknosis of

the nuclei was evident within 1 h and DNA fragmentation as detected by TUNEL staining was evident by 6 h after nsPEF treatment. In addition we demonstrated that nanoelectroablation stimulated an immune response that inhibited secondary tumor formation in an allograft model. Other groups have also reported that apoptotic cells can trigger an immune response[28, 29] and we are continuing to study this exciting observation.

Another more naturally occurring skin cancer model is the basal cell carcinoma (BCC) Ptch1+/-K14-Cre-ER p53 fl/fl mouse model developed by Ervin Epstein[30, 31]. These BCCs develop naturally on the skin of mice that were treated as pups with ionizing radiation. We treated 27 of these BCCs and all began to shrink within a day after treatment. After 4 weeks they were 99.8% ablated if the electrode matched the tumor size[32]. Pyknosis was evident within 2 days after nsPEF treatment and DNA fragmentation as detected by TUNEL staining was also evident post treatment.

Our ultimate goal is to use nanoelectroablation to treat human carcinomas. Towards that goal, we have been treating xenograft tumors created by injecting human pancreatic carcinoma cells beneath the skin of immunosuppressed mice. After treating dozens of human pancreatic tumors with a range of pulse numbers, we determined that 500 pulses of 100 ns, 30 kV/cm were sufficient to ablate these tumors with a single treatment. In addition, we conducted a long-term study of 19 mice in which a single tumor was nanoelectroablated and observed over a period of at least 300 days[33] (fig.1). No tumor recurrence was detected. In contrast, untreated control tumors continued to grow until we were forced to sacrifice the mouse within 110 days.

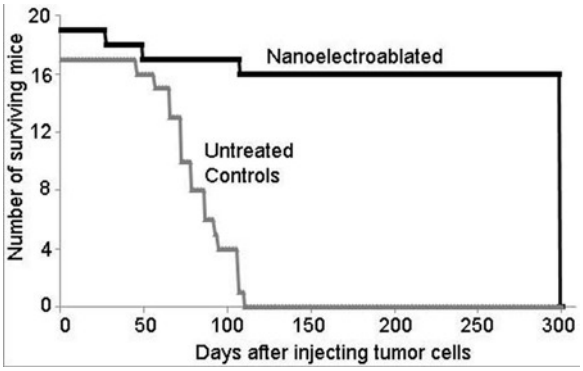

Figure 1. Survival curve for both mice with nanoelectroablated human pancreatic tumors as well as untreated controls. Capan-1 pancreatic tumors were induced in these mice on day 0 and treated with 500-1000 pulses (30 kV/cm, 100 ns) when they were 4 mm in diameter. Treated mice were sacrificed at 300-330 days after tumor injection to conduct histology on treated skin regions. (reproduced with permission from *Int. J. Cancer doi: 10.1002/ijc.27860*)

Ca^{2+} increases

One of the first cellular changes in response to nsPEF is an increase in intracellular Ca^{2+}[25, 34-37]. This increase is due to both Ca^{2+} influx through the plasma membrane and release from the ER. Recent work has revealed that Ca^{2+} entry across the plasma membrane is localized to the region near the electrodes[36] and some quantitative fura-2 studies have shown that the Ca^{2+} increases linearly by 8-10 nM per 1 kV/cm until a critical concentration between 200 and 300 nM at which Ca^{2+}-induced Ca^{2+} release is initiated through IP_3 receptors in the ER[37]. This intracellular Ca^{2+} increase will probably trigger other downstream steps in the apoptotic response. It appears to be required for reactive oxygen species generation as discussed next and also may be involved in the mitochondrial membrane potential decline[22].

Reactive Oxygen Species (ROS) Generation

It was found nearly 20 years ago that pulsed electric fields in the microsecond domain trigger ROS generation[38, 39]. Pakhomova and colleagues recently reported similar oxidative effects of nsPEF[40]. ROS generation begins within a minute after nsPEF exposure and continuously increases for over an hour. We have also been studying this and find that ROS generation is Ca^{2+}-dependent. ROS stimulation was greatest under normal Ca^{2+} conditions. When BAPTA-AM was used to chelate intracellular Ca^{2+}, ROS production was reduced by a factor of two. However, since extracellular Ca^{2+} can enter through the nanopores induced in the plasma membrane by the pulses we would expect it to exceed the buffering capacity of the internal BAPTA. The only way to prevent this is to buffer extracellular Ca^{2+} as well. When EGTA is used to chelate extracellular Ca^{2+} alone, ROS production is also even lower than that when intracellular Ca^{2+}

was chelated. However, when both intracellular and extracellular Ca^{2+} were chelated, the ROS level dramatically dropped to 90-94% of control levels (fig. 2). These results suggest that Ca^{2+} plays an important role in the nsPEF-triggered increase in ROS in human pancreatic carcinoma cells.

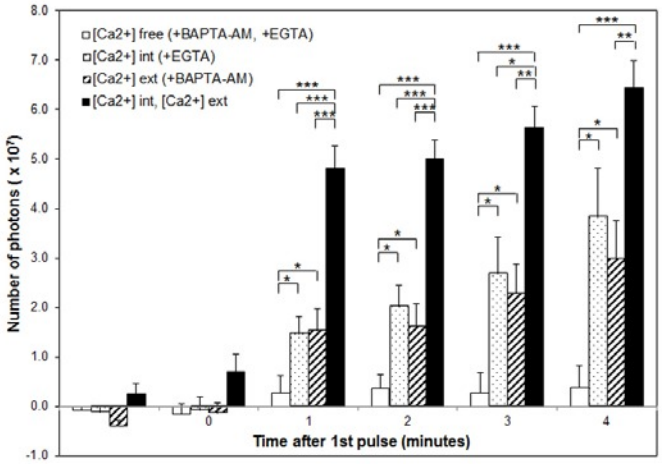

Figure 2. Ca^{2+} dependence of ROS generation. Cultured BxPC-3 cells were stimulated with 100 30 kV/cm, 100 ns pulses at 5 Hz. Chelating either [Ca]ext (dotted bars) or [Ca^{2+}]int (strip bars) resulted a lower ROS production. Chelating both [Ca^{2+}]ext and [Ca^{2+}]int show a strongest decrease in the fluorescence, indicating that Ca^{2+} is substantial for generating ROS in the process. Each condition is repeated with at least four independent experiments; at least two individual cells are measured in each experiment. Bars are the mean, error bars are the standard error of the means. * $p<0.05$, ** $p< 0.01$, ***<0.001.

4. PROGRESS TOWARDS HUMAN THERAPY

We are developing the NanoBlate® 100 ns pulse generator for the ablation of soft tissue. This pulse generator can produce up to 20 kV pulses at 2 pps. We are developing electrodes to deliver these pulses to skin lesions and internal organs. Our first clinical trial focused on treating human basal cell carcinomas (BCC). We are determining the pulse number needed to nanoelectroablate these malignant lesions. Our preliminary results look very encouraging with the complete scarless ablation of BCCs after a single 2 minute-long treatment (fig. 3).

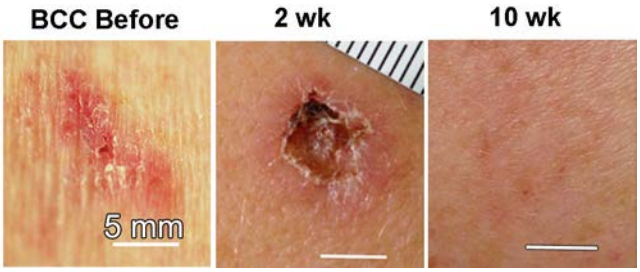

Figure 3. One cm-long basal cell carcinoma treated with 100 pulses of 100 ns, 30 kV/cm. Three photos of the lesion show it before treatment as well as 2 and 10 weeks after treatment. The scale bar in each photo is 5 mm long.

5. CONCLUSION

Nanoelectroablation with 100 ns pulses has many advantages over other ablation techniques such as irreversible electroporation and radio frequency ablation (RFA). It is the only non-thermal ablation that triggers apoptosis rather than necrosis. The slower apoptotic cell death allows more time for the immune system to be activated and to remove the dead tissue. At least for skin this leaves no scar in the treated region. Another advantage is the very short treatment time of a few minutes compared to RFA that often takes 20 min. This only disadvantage of nanoelectroablation is the very high electric field required. This limits the electrode spacing to about 1 cm so that larger tumors will require multiple treatments after repositioning the electrodes in order to treat the entire tumor.

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