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Long Term Survival of Mice With Hepatocellular Carcinoma after Pulse Power Ablation with Nanosecond Pulsed Electric Fields

Novel therapies are needed for treating hepatocellular carcinoma (HCC) without recurrence in a single procedure. In this work we evaluated anti-neoplastic effects of a pulse power ablation (PPA) with nanosecond pulsed electric fields (nsPEFs), a non-thermal, non-drug, local, regional method and investigated its molecular mechanisms for hepatocellular carcinoma tumor ablation in vivo. An ectopic tumor model was established using C57BL/6 mice with Hepa1-6 hepatocellular carcinoma cells. Pulses with durations of 30 or 100 ns and fast rise times were delivered by a needle or ring electrode with different electric field strengths (33, 50 and 68 kV/cm), and 900 pulses in three treatment sessions (300 pulses each session) or a single 900 pulse treatment. Treated and control tumor volumes were monitored by ultrasound and apoptosis and angiogenesis markers were evaluated by immunohistochemistry. Seventy five percent of primary hepatocellular carcinoma tumors were eradicated with 900 hundred pulses at 100 ns pulses at 68 kV/cm in a single treatment or in three treatment sessions without recurrence within 9 months. Using quantitative analysis, tumors in treated animals showed nsPEF-mediated nuclear condensation (3 h post-pulse), cell shrinkage (1 h), increases in active executioner caspases (caspase-3 > -7 > -6) and terminal deoxynucleotidyl transferase dUTP nick-end-labeling (1 h) with decreases in vascular endothelial growth factor expression (7d) and micro-vessel density (14d). NsPEF ablation eliminated hepatocellular carcinoma tumors by targeting two therapeutic sites, apoptosis induction and inhibition of angiogenesis, both important cancer hallmarks. These data indicate that PPA with nsPEFs is not limited to treating skin cancers and provide a rationale for continuing to investigate pulse power ablation for hepatocellular carcinoma using other models in pre-clinical applications and ultimately in clinical trials. Based on present treatments for specific HCC stages, it is anticipated that nsPEFs could be substituted for or used in combination with ablation therapies using heat, cold or chemicals.

Key words: Electric fields; Apoptosis; Angiogenesis; Non-thermal ablation; Caspases; TUNEL; Micro-vessel density.

Introduction

The liver is an enormously complex, essential-for-life organ. It is comprised of a number of cell types, with the major type being hepatocytes, which are affected...
New treatments are needed for very early, early and intermediate stages, with recurrences and metastasis as major problems. Local control therapies and chemotherapy have shown their ability to downstage tumor severity, but are primarily palliative (12). Chemoembolization and selective internal radiation therapy have shown modest (11). Other methods such as transcatheter arterial embolization (TAE) or transcatheter arterial chemoembolization. Currently there is no standard treatment for patients with non-resectable HCC (7).

Stages O and A include patients with 1-3 nodules ≤ 3 cm in the presence or absence of portal hypertension and/or other characteristics of liver dysfunction. These include ~30% of patients with HCC, a significant population. Chemo-therapeutic agents, which are treatment options for many advanced stage HCC, are usually not effective due to resistances related to changes in expression of target proteins and altered drug metabolism (7). Doxorubicin is the traditional reference standard for evaluating new agents. Patients taking sorafenib, a new multi-targeted kinase inhibitor targeting cell proliferation and angiogenesis pathways, experience higher rates of stable disease, but response rates were relatively modest (11). Other methods such as transcatheter arterial chemoembolization and selective internal radiation therapy can downstage tumor severity, but are primarily palliative (12). Local control therapies and chemotherapy have shown their limits, with recurrences and metastasis as major problems. New treatments are needed for very early, early and intermediate disease stages that avoid recurrences of intrahepatic nodules and multiple procedures.

There are several ablation strategies available for HCC and one of the most commonly used new procedures is RFA. Although RFA has shown effectiveness in tumor ablation, there are several potential major complications including monitoring the ablation zone, heat-sink effects, pneumothorax, hemoperitoneum, portal thrombosis, capsular hematoma, infections and neoplastic seeding (7, 13). A newer ablation therapy, irreversible electroporation (IRE), involves the application of conventional electroporation (EP) with very high electric field intensity to induce cell death by necrosis (14). IRE as a treatment for HCC or other cancers has the advantage of being non-thermal, thereby avoiding heat related side effects. Although there is some evidence to suggest that ablation zones may also exhibit at least limited numbers of apoptotic cells (15, 16), suggested advantages of IRE include a distinct margin between treated and viable tissue and little impact on collagen network and blood vessels (16). A disadvantage of the procedure is the need for neuromuscular blockade to prevent intense muscle contractions during IRE treatment.

Nanosecond Pulsed Electric Field (nsPEF) Technology

Another new technology, pulse power ablation (PPA) using nanosecond pulsed electric fields (nsPEFs), decreases the pulse duration and greatly increases the electric field compared to conventional electroporation (17-21). The enabling technology for PPA is the pulse power technology, where electric energy is stored at low power levels, and released in a single pulse or in a burst of high electrical power. Like IRE, this non-thermal therapy avoids heat related injury and side effects. Particularly, electrical pulses in the nanosecond range, when applied to cells and tissue, have been shown to alter cell functions dependent on pulse duration, amplitude and pulse number. The effect of the pulse duration on cell functions can be understood by using a simple electrical model for a biological cell (17). It predicts that reducing the duration of applied electrical pulses to values below the charging time of the outer cell membrane (which is on the order of 100 ns for mammalian cells) causes a strong increase in the probability of electric field interactions with intracellular structures due to displacement currents. For electric field amplitudes exceeding MV/m, such pulses are also expected to allow access to the cell interior through conduction currents flowing through the permeabilized plasma membrane. In both cases, limiting the duration of the electrical pulses to nanoseconds ensures only non-thermal interactions of the electric field with subcellular structures. This intracellular access is believed to allow manipulations of cell functions. Several experimental studies support this hypothesis and suggest that it is possible to selectively alter the behavior and/or survival of cells (22).

Esser et al. (23) used a multicellular system model with irregularly shaped liver cells and a multiscale liver tissue model to determine whether pore-based effects could possibly explain nsPEF-induced apoptosis. Other studies by the Weaver group (24, 25) lead to the concept of supra-electroporation, which creates an extraordinary numbers of nano-sized pores.
(about 1 nanometer) in all cell membranes, including those of intracellular organelles, allowing only small molecular transport across cell membranes. Nanosecond pulses are believed to affect subcellular structures, either through the displacement current flowing during the early part of the pulse, or through the conduction current after the conductance of the plasma membrane is increased through poration. Consequently, the electric field correlated to the current density in the cytoplasm will affect subcellular membranes in the mitochondria and endoplasmic reticulum. It is possible that this phenomenon could explain a number of observations for intracellular effects that could lead to cell death by apoptosis which has been shown to be coincident with nsPEF-induced cell death (20).

Effects of nsPEFs to decrease the mitochondria membrane potential have been reported (26, 27), which could ultimately lead to cytochrome c release (26, 27). As suggested by Weaver (28), it is possible that this could be due to nsPEF-induced opening the mitochondria permeability transition pore complex (MPTP) and/or mitochondrial membrane voltage-dependent anion channels. However, while effects of nsPEF have been shown to decrease the mitochondria membrane potential (26, 27) there are no studies that specifically demonstrate effects of nsPEFs on MPTP.

Observed nanosecond pulsed effects at moderate electric fields include intracellular release of calcium and enhanced gene expression, which could have long term implications on cell behavior and function. At increased electric fields, the application of nanosecond pulses induces a type of programmed cell death, apoptosis, in biological cells. Since the initial discovery that nsPEFs trigger apoptosis signaling pathway(s) (19, 20), there have been significant efforts made to determine the exact mechanism of this process.

One of the possible reasons for apoptosis induction was assumed to be extensive calcium release, caused by nanosecond pulses (29). Calcium at high concentration is known to induce apoptosis. However, in experiments on Jurkat and HL-60 cells (21) it was found that chelation of calcium in the extracellular media with EGTA and in the cytoplasm with BAPTA-AM had little or no effect on caspase activation, suggesting that calcium was not required for nsPEF caspase activation, but is likely involved in other nsPEF-induced apoptosis mechanisms. It remains to be determined if the observed calcium mobilization, which can come through the plasma membrane or from intracellular stores (the endoplasmic reticulum), is a primary cause or the result of nsPEF-induced cell death.

The production of reactive oxygen species and subsequent DNA damage has also been proposed as a possible effect causing apoptosis (30). There is evidence that nsPEF stimulation with multiple, intense pulses cause damage to DNA or other critical proteins. Studies by comet assay have shown that significant DNA damage does occur very quickly after treatment (90 seconds post treatment) with nanosecond pulsed electric fields (31).

In addition to apoptosis, changes in microvessel density caused by nanosecond pulses have been observed, using immunohistochemistry. Tumor growth depends on the availability of nutrients which, after the disruption of vasculature, requires the formation of new capillaries in a process known as angiogenesis. Endothelial cells forming capillaries can be detected by using antibodies to the endothelial cell marker, CD34 (32). The endothelial cell density in both nsPEF-treated and untreated sections from 5 different melanomas was recorded and an average reduction of more than 90% in CD31 expression in nsPEF-treated tumors was found. This suggests that the microcirculation of treated tumors is severely reduced and this should lead to necrosis and tumor shrinkage.

It needs to be pointed out that in spite of the high electric fields which are generated in cells and tissue, due to the extremely short duration of these high power pulses, thermal effects, similar as for IRE, are nonexistent (33-35). Like IRE, treatment zones are clearly defined; however, in contrast to IRE, contractions during nsPEF treatments are absent or significantly minimized (36). However, this can depend on how the animal is grounded, the position of the tumor, the placement of the electrodes and other aspects that we have not systematically analyzed. Nevertheless, applications of nsPEF target therefore two important therapeutic mechanisms: apoptosis induction and anti-angiogenesis, making it a potentially significant addition to HCC treatment arsenal.

In this study, PPA with nsPEFs using various numbers of square wave pulses with durations of 30 or 100 ns, rise times of ~4 ns and average electric fields as high as 68 kV/cm are applied to ectopic Hepa1-6 HCC in an in vivo murine model. We show that nsPEF eliminated as many as 75% of Hepa1-6 HCC tumors without recurrence for as long as 9 months. These results indicate that nsPEF impinges on at least two cancer hallmarks and therapeutic targets: apoptosis induction leading to anti-angiogenesis as well as other anti-vascular effects. nsPEFs can serve as independent treatments for precisely targeted tissue ablation or to act as adjuncts to other anti-tumor modalities.

Materials and Methods

Cell Line

Hepa1-6 murine hepatoma cells were purchased and culture according to recommendation from ATCC at 37°C with 5% CO₂ and 95% air.
Marine Tumor Model

Animal experiments were approved by the Old Dominion University Institutional Animal Care and Use Committee. Tumor were induced by subcutaneous injection of Hepa1-6 cells (1 × 10^6 in 100 μl PBS) in the flank of female C57BL/6 mice (4 wks, ~20 g; Charles River) Tumors became palpable (0.3 cm-0.5 cm in diameter) 7 days after inoculation.

Pulse Delivery Assembly and nsPEF Treatment

The high voltage pulses were supplied by a Blumlein line pulse generator as previously described (37). More basic information on nanosecond pulse generators can be found in (38). The pulses were delivered to tumors by a needle array or ring electrode (37). All materials were made using surgical stainless steel. The needle electrode is a 4+1 needle array with 4 grounded perimeter needles forming a square and a high voltage biased center needle. For the ring electrode, a coaxial ring applicator replaced the grounded needle electrodes. The inner ring diameter was 8 mm, the same as the diagonal distance of the needle array. Both electrode designs completely surround tumors (0.3-0.5 cm).

Figure 1 shows the electrode arrangements and the electric field distributions in both electrode systems. Clearly, the electric field distribution is inhomogeneous; more in the 4+1 needle configuration, less in the needle-ring configuration. The electric field intensity, which reaches values of up to 230 kV/cm at the needle electrode surfaces, decreases in the needle to ring electrode system to values on the order of 40 kV/cm (with lowest values close to the ring). In the 4+1 needle array it decreases from the center electrode, reaches a minimum of about 40 kV/cm at two-thirds of the distance from center to the outer needles, and increases again to almost 90 kV/cm at the surface of the outer needle electrode. Of course, whereas the electric field distribution in the ring electrode is rotationally symmetric about the center electrode, in the 4+1 needle array it shows maxima at the radial positions, which are defined by the location of the outer electrodes. In the following, we have used the average electric field intensity, which we defined as the voltage divided by the distance between inner and outer electrodes, as a measure of the electric field intensity.

A problem which we encountered when applying very high voltages (~70 kV/cm) between the needles, was surface flashover, the formation of plasma channels on the surface of the tumor. A way to avoid this flashover, is by using an insulating fluid with high dielectric strength. In the case of the needle to ring configuration, the ring, which contacts and seals a perimeter of the tumor, was filled with water to prevent pulse breakdown at high electric fields.

Survival Study

Control groups were sham-treated and nsPEF groups were given pulses of 30 ns or 100 ns with various electric fields, pulse numbers and treatment sessions. Specific treatment conditions are listed in the legend to Figure 2. Mice were followed for at least 269 days after treatment except those humanely euthanized with excessive tumor burden (2 cm).

Tumor Measurement

Tumor volumes were determined by ultrasound (Visual Sonics Vevo 770, model 708 scan head at 55 MHz, Visualsonics Inc.), which agreed with volumes measured by tumor length and width using a Vernier caliper (39).
Mice were treated with 900 pulses at 100 ns and 68 kV/cm. Tumors were removed at indicated times, fixed in formalin and prepared for immunohistochemistry. Tissue slices were incubated with rabbit antibodies specific to phospho-Bad or active executioner caspases (Cell Signaling Technologies) as indicated and then incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG. The numbers of positive cells were counted from a total of 300 cells in non-overlapping frames. Each experiment was repeated twice.

Measurement of Micro Vessel Density (MVD) With CD34 and Vascular Endothelial Growth Factor (VEGF) Expression

Tumors in 4 mice were treated with 900 pulses of 100 ns with a needle array electrode at 68 kV/cm. Tumors from another 4 mice served as controls. Tumors were removed 0, 7, 14 and 21 days after treatment and prepared for immunohistochemistry as indicated above. The primary antibody was polyclonal rabbit anti-CD34 (BioVision) or a mouse anti-VEGF primary antibody (Santa Cruz Biotechnology). Sections were covered with HRP-conjugated goat anti-rabbit IgG for CD34 or goat anti-mouse IgG for VEGF (Invitrogen). Nonspecific staining was determined with secondary antibody only. The chromogen diaminobenzidine provides brown deposits in situ.

Statistical Analysis

Results are shown as mean ± SE. One-way ANOVA was used to evaluate the difference of means between nsPEF treated group and control group. The statistical differences were considered at the probability level (p value) of less than 0.05.

Results

Long Term Survival of Mice Treated for HCC With nsPEFs

We had previously treated B16f10 melanoma tumors with nsPEFs using pulse duration of 300 ns (31, 34, 35, 39). We attempted to use pulses with shorter duration, 30 ns and 100 ns in thus study, based on the theory that shorter pulses with fast rise time provides advantages for targeting intracellular structures, which are hypothesized to be more effective for inducing programmed cell death, most likely apoptosis.

TUNEL Assay

DNA damage was detected using ApopTag Red in Situ, (Chemicon) based on terminal transferase mediated dUTP nick end labeling (TUNEL) assay according to the manufacturer’s instructions. TUNEL, positive tumor cells showed red cytoplasmic fluorescent halos. Nuclei were stained blue with DAPI. The merged images revealed purple TUNEL positive cells as opposed to cells with only blue nuclei. Nuclei (100) were analyzed as described above. The positive cells were outlined and counted by software Image J and expressed as mean ± SD.

Analysis of Bad Phosphorylation and Activation of Executioner Caspases In Vivo

Mice were treated with 300 pulses at 100 ns and 68 kV/cm. Tumors were removed at indicated times, fixed in formalin and prepared for immunohistochemistry. Tissue slices were incubated with rabbit antibodies specific to phospho-Bad or active executioner caspases (Cell Signaling Technologies) as indicated and then incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG. The numbers of positive cells were counted from a total of 300 cells in non-overlapping frames. Each experiment was repeated twice.

Short Term Tumor Morphology Changes After nsPEF Treatment

Mice were treated with 900 pulses at 100 ns and 68 kV/cm with a needle array electrode. The center electrode pierced the center of the tumor and the tumors were confined within the boundaries of the outer needles of ring. Tumors were removed at various times after treatment for histological and immunohistochemical analysis. Tumors were fixed in 10% neutral buffered formalin prior to paraffin processing. Tumor sections (5 μm) were deparaffinized in xylene, rehydrated, stained with H&E and assessed microscopically for abnormal cell morphology. Slices of tumors were taken randomly except for exclusion of the center and the outer boundaries where the electric fields were highest. Nuclei (100) were randomly selected and outlined in 10 non-overlapping fields of each section at 200× magnification. The nuclear areas were calculated by MATLAB software and summed as the mean ± SD for nuclear area calculation and statistical analysis.

Figure 2: NsPEF parameter investigation: The following treatment conditions were tested: 1. Control: sham nsPEF treatment (n = 8); 2. 30 ns-R: 300 pulses, 30 ns, 68 kV/cm, three treatments on alternate days, needle electrode, (n = 7); 3. 30 ns-N: 300 pulses, 30 ns, 68 kV/cm, three treatments on alternate days, needle array electrode (n = 8); 4. 100 ns-H: 300 pulses, 100 ns, 68 kV/cm, three treatments on alternate days, needle array electrode, (n = 6); 5. 100 ns-M: 300 pulses, 100 ns, 50 kV/cm, three treatments on alternate days, a needle array electrode (n = 6); 6. 100 ns-L: 300 pulses, 100 ns, 33 kV/cm, three treatments on alternate days, needle array electrode (n = 6); 7. 100 ns: 900 pulses, 100 ns, 68 kV/cm, single treatment, needle array electrode (n = 6); 8. 30 ns-C: 30 ns-C: 300 pulses, 30 ns pulse with corona effect, three treatments on alternate days (n = 7).

TUNEL Assay

DNA damage was detected using ApopTag Red in Situ, (Chemicon) based on terminal transferase mediated dUTP nick end labeling (TUNEL) assay according to the manufacturer’s instructions. TUNEL, positive tumor cells showed red cytoplasmic fluorescent halos. Nuclei were stained blue with DAPI. The merged images revealed purple TUNEL positive cells as opposed to cells with only blue nuclei. Nuclei (100) were analyzed as described above. The positive cells were outlined and counted by software Image J and expressed as mean ± SD.
Figure 2 shows Kaplan-Meyer representation of nsPEF conditions that treated ectopic Hepa1-6 hepatocellular carcinoma (HCC) in mice in vivo. Several treatment conditions were tested. Both ring (R) and needle (N) electrodes were used. Treatment regimens included low (L, 33 kV/cm), medium (M, 50 kV/cm) and high (H, 68 kV/cm) electric fields with 30 ns or 100 ns durations. Treatment sessions included three treatments on alternate days or a single treatment. The three day regimen includes 300 pulses at 30 or 100 ns each day. The repetition rate is always 1 Hz. In an attempt to eliminate the tumors with one treatment, a final treatment consisted of 900 pulses at 100 ns and 68 kV/cm requiring 15 minutes to complete at 1 Hz. Specific treatment combinations are indicated in the legend to Figure 1. Untreated mice survived for 12-17 days before tumor burden (2 cm) required euthanasia according to our IACUC protocol. Specific treatment combinations are indicated in the legend to Figure 2. In general, treatments fell into two general efficacy zones of greater than 75% survival or less than 40% survival for 260-269 days after treatment. The most effective treatments included the single treatment with 900 pulses at 100 ns and 68 kV/cm (number 7) and three treatments with 300 pulses at 100 ns and 68 kV/cm on each of three alternate days (number 4). Both of these conditions used the needle electrode. For the latter regimen, similar results were found in a larger trial with 76.5% survival (13/17). So for most effective treatments, high electric fields were required at 100 ns with 900 pulses either in a single session or accumulated over three treatment days.

Less effective conditions fell into several categories, which included either 30 ns at any of the three electric fields or 100 ns pulses with electric fields that were not 68 kV/cm. These ineffective treatments included those from Figure 1 as numbers 2 and 3 with 30 ns pulses at 68 kV/cm as well as 5 and 6 with 100 ns pulses at 33 or 50 kV/cm. Another ineffective treatment was number 8 when a corona discharge was used. A corona discharge is generated in the tissue when the pulsed electric field is high enough to form a conductive region with ionized gases generated around the electrode. In this study, strong corona discharges can be observed when the center electrode is positively charged.

**Tumor Regression After nsPEF Treatment**

Figure 3 shows tumor volumes visualized with ultrasound of tumor growth in control and regression mice treated with nsPEFs. These representative images show a regimen with 300 pulses at 100 ns and 68 kV/cm on three alternate days beginning on day 0 with over a 14 day period. Treatment began when tumors were about 0.4 cm. Treated and control ultrasound images are shown in the two top panels and calculated tumor volumes are shown in the graphic representation at the bottom. Tumors disappeared with 100 ns pulses to nearly non-detectable levels 14-21 days after the first treatment in 6 of 8 mice. For 30 ns treatments tumor regression was slower and was only effective in 25% of mice (not shown).

**Rapid Nuclear Condensation After Treatment With nsPEFs**

Figure 4 illustrates time-dependent effects on short-term tumor morphology (left panels) with a focus on nuclear area (graph) after a single treatment with 300 pulses at 100 ns and 68 kV/cm using the needle electrodes. The H&E staining revealed Hepa1-6 tumor ultra-structure and nuclear changes.
after treatment. Control tumor cells featured round, regularly light blue stained nuclei with prominent nucleoli. The cytoplasm was characteristically purple and homogeneous. Treated tumor nuclei featured highly condensed chromatin, segregating into sharply defined bodies (nuclear pyknosis). Treated cells exhibited shrinkage and cytoplasmic condensation with disintegrating cell connections and disappearance of cord-like supporting structure on which tumor cells extended. Individual cells became multi-angular with decreased nuclear/cytoplasmic ratios and enlarged extracellular spaces. The data show a time-dependent decrease in tumor nuclei size to less than 20% of their original volume in 24 hours. The most significant reduction occurs in the first 3 hours after treatment.

**Rapid Appearance of TUNEL Positive Cells After nsPEF Treatment**

Figure 5 shows an analysis of control and treated tumor nuclei using TUNEL to indicate DNA fragmentation damage, which is often used as a marker for apoptosis (40). The fluorescent microscope images shows tumor cell nuclei stained bluish-purple with DAPI and TUNEL positive cells with DNA damage as reddish orange cytoplasmic halos. The merged images revealed apoptotic cells with pinkish nuclei and non-apoptosis cells as purple cells. A graphic representation of TUNEL positive cells is shown at the bottom. The data indicate a time-dependent increase in TUNEL positive cells within the first three hours after treatment, such that as many as 50-60% of cells are TUNEL positive. Statistically significant increases in TUNEL positive cells occurred during the first 9 hours with a peak at 3 h after nsPEF treatment. However, this response is transient and the numbers of TUNEL positive cells decrease between the third and sixth hour after treatment, such that 24 hours after treatment few cells show positive responses.

**NsPEFs Induce Caspase Activation but not Bad Phosphorylation**

The results in Figure 6 analyze treated and fixed tumor slices with antibodies for active executioner caspases-3, -6 and -7 as well as for phosphorylated Bad. Phosphorylated Bad was not present at any time tested. For each isozyme, significant increases in caspase-positive cells peaked at three hours after treatment, with caspase-3 showing the highest and caspase-6 the lowest percentages. Significant numbers of caspase positive cells remained elevated for 6 to 7 hours after treatment. While the presence of active caspase-3 and -6 decreased progressively from 3 to 7 hours, active caspase-7 declined more slowly, suggesting differential turnover for active caspase isoforms. In no tissue slices did caspase positive cells amount to greater than 50% of total cells and caspases-6 and -7 accounted for an average of 20% and 30%, respectively. It was not possible in this study to determine whether some cells contained only one or more than one active caspase isozyme.
NsPEF Treatment Results in Down Regulation of Angiogenic VEGF

PPA with nsPEFs has been shown to have effects on tumor vasculature (34, 35, 39). As an important therapeutic target, we investigated effects on vascular endothelial cell growth factor (VEGF), the most ubiquitous pro-angiogenic factor (Figure 7) and a downstream VEGF respondent CD34, a common endothelial micro-vessel density (MVD) marker (Figure 8). Figure 7 shows representative IHC staining for VEGF in control and treated Hepa1-6 tumors in the top two panels and a graphical representation of VEGF positive cells at the bottom. After nsPEF treatment there was a time-dependent decrease VEGF, while VEGF exhibited a time-dependent increase in control cells. Three weeks after treatment, VEGF decreased 83% compared to the day of treatment, decreases 7-fold compared to control levels and decreased >5-fold compared to day 0.

Figure 7: Effect of nsPEFs on VEGF expression by Immunohistochemistry: Mice were treated with 100 pulses at 100 ns and 68 kV/cm with a needle array electrode and repeated 3 times on alternate days. Two tumors on each mouse were selected randomly for control or pulse treatments. Tumors were removed on indicated days after nsPEF treatment and prepared for immunohistochemistry. Tissue slices were incubated with an antibody to VEGF. The appearance of VEGF is indicated by cells with brown color due to staining with diaminobenzidine. Brown vessels were counted and summarized as the mean ± SD based on 3 individual slides from each of two tumors from same mouse at each time point. Statistical significance is at p < 0.05.

NsPEF Treatment Results in Down Regulation of a Microvascular Target

Figure 8 show IHC staining demonstrating the presence of CD34 as indicated by the stained, brown-colored cells within the top panels and a quantitative analysis of the results in the bottom. In untreated controls CD34 increased more than 4-fold after 3 weeks. In contrast, CD34 decreased 75% from the day of treatment and more than 8-fold less that the untreated control on day 21.

Discussion

This study demonstrates successful treatment of Hepa1-6 HCC using pulse power ablation, a non-thermal, non-drug, local therapy in an ectopic murine model. Optimal treatments included 76.5% tumor-free survival for nearly 9 months. The other 23.5% survived an average of 43 days, three times longer than controls. This significant but partial efficacy was probably due to inadequate exposure of all HCC cells to sufficient electric fields. This assumption is based on the electric field-dependent cell death of both cells in culture and in this study using lower electric fields. These results clearly demonstrate that nsPEF tumor treatment is not restricted to treating skin cancers, such as B16f10 melanoma (31, 34, 35, 39, 41). Interestingly, the difference between 76.5% and 37.5% survival was an increase in electric field of 18 kV/cm, suggesting that further electric field increases would result in complete elimination. In vitro studies also indicate nsPEFs can eliminate a number of cell types including Jurkat, HL-60, p53 wild-type and null HCT-116, B16f10 melanoma and E4 squamous cell carcinoma (20, 21, 26, 27, 42). In all of these cell types, signatures of apoptosis were coincident with cell death indicating that nsPEFs induces cell death at least in part by apoptosis induction. However, caspase activity was not observed in all cells and when present it was transient; therefore, other forms of programmed cell death must be present. This supposed caspase-independent cell death could exhibit characteristics of necrosis, but this was not tested. Given that the tumor mass cannot be removed before the membrane potential of cells is completely dissipated, later
stages of cell death are likely necrosis. The induction of programmed cell death appears to be due to non-thermal, electric field interactions with the outside and inside of cells and tissues and not heat.

The elimination of HCC tumors in vivo demonstrates nsPEF mechanisms of action that address two major cancer hallmarks and well-characterized therapeutic targets including apoptosis induction and anti-angiogenesis. Anti-vascular effects of nsPEFs are effective to limit tumor blood supply. Hepatocyte apoptosis is considered to be a major step in most forms of liver injury. Chronic cell turnover provides the bases for apoptosis initiating mutations and chronic pro-apoptosis pressure promotes mechanisms to evade apoptosis (5). Hepa-1-6nsPEF-induced cell death occurs, at least in part, in the presence of apoptosis as indicated here by typical morphological changes, which are caused, at least in part, by activation of caspases. Thus, nsPEF reverses a primary initiator of tumorogenesis in HCC. Interestingly, the peaks of active caspase- and TUNEL-positive cells occur at nearly the same time in similar numbers of cells after nsPEF treatment. However, it is not possible to tell from these studies if they coexist in the same cells. In B16f10 melanoma, peaks of TUNEL-positive cells occurred before peaks of caspase activation, suggesting caspase-independent cell death mechanism(s) (39). Alternatively, like caspase-activity, TUNEL was transient, suggesting the possibility that DNA damage could be related to caspase activity. Since apoptotic DNA fragmentation is a relatively late, caspase-dependent event, the presence of TUNEL-positive cells in response to nsPEFs may not be an accurate indicator of numbers of apoptotic tumor cells. Thus, apoptotic cell death in tumors (this study; 39) and in ischemic and reperfused liver (5, 43) or heart (44), where massive cell death occurs without immediate phagocytosis, may not be fully comparable to cell death during normal development or deletion of immune cells that recognize self antigens where inflammation does not occur. Like these other tissues, apoptotic cell death in tumors may coexist with other forms of cell death such as necrosis, secondary to apoptosis, necrosis or outright necrosis. This is especially relevant to liver where apoptosis is closely associated with inflammation and fibrosis (5). While other mechanisms of cell death in response to nsPEFs have not been scrutinized in vitro, apoptosis markers appear in nsPEF treated tumors in vivo and cells in vitro.

Sustained angiogenesis is a second cancer hallmark and cancer therapeutic site that is targeted by nsPEF ablation. Targeting non-transformed, less resistant endothelial cells that support tumors may have therapeutic advantages (45) that do not involve recurrences and that may prevent metastasis. VEGF, a potential angiogenic factors required for the angiogenic switch, was shown to decrease after treatment with nsPEFs. Microvascular density (MVD) markers, which are downstream respondents of VEGF, have been shown to positively correlate with recurrences (46). CD31 was significantly decreased here as well as in B16f10 melanoma (39). This indicates that anti-angiogenesis is a common target of nsPEFs in skin, liver and likely in other tumors. While it may be argued that elimination of tumors removes stimuli for vasculogenesis, given that non-transformed endothelial cells are unlikely to display characteristics of malignant transformation, they are excellent cancer therapeutic targets (45). This is especially true for larger HCCs (≥2 cm), which are hypervascular with well-developed arteries and sinusoids (47).

The question arises as to which HCC stages would be treatable with nsPEFs. These studies, which involved treating relatively small ectopic HCC and studies of nsPEF-induced cell death in normal porcine liver (36) begin to answer this question. It is anticipated that by minimally invasive procedures with laparoscopy, ultrasound guidance that circumvent major surgery and catheter electrodes, nsPEF ablation can substitute for or complement other local ablation treatments. Given that other local ablation methodologies have shown limitations and that targeting one molecular component does not necessarily suppress HHC progression, the inclusion of nsPEF ablation could provide significant advantages for HCC patients. Based on the data presented here and dependent on successful treatment in orthotopic HCC models, which are in progress, it is likely that nsPEF ablation could treat the same HCC stages as those treated with other non-surgical therapies such as RFA, cryoablation, microwave coagulation, percutaneous ethanol or acetic injection or transarterial embolization. NsPEF ablation has significant advantages over other local regional treatment modalities including non-thermal ablation, a well-defined treatment zone, no requirement for neuromuscular blockers and multiple therapeutic targets. In addition, nsPEF effects do not depend on the size or orientation of the cell. In contrast, IRE treatments conditions must be set for smaller cells since larger and more extended cells require lower electric fields for plasma membrane perturbation. Since tumors are generally heterogeneous within and among tumor types with different morphologies, a wide variety of tumor types should be successfully treatable with nsPEFs (23). This is specifically exemplified here for Hepa1-6 HCC and elsewhere for B16f10 melanoma (31, 34, 35, 39, 41) and in AsPC-1 pancreatic and a basal cell carcinoma (48).

NsPEF ablation targets two well-characterized cancer hallmarks: apoptosis evasion and sustained angiogenesis. This treatment can eliminate HHC tumors in vivo by activating caspases to bypass pro-survival signals and promote apoptosis, which not only eliminates tumors, but also promotes healing and tissue regeneration (49, 50) in the former cancer microenvironment. NsPEF ablation also thwarts angiogenesis by decreasing the angiogenic switch initiator, VEGF and diminishing downstream microvascular endothelial components, CD34. Finally, nsPEF ablation immediately
permeabilizes small blood vessel structures (31, 35, 36), mimicking antivascular therapy (51, 52). The studies here provide a clear rationale for continued development of nsPEF ablation as a therapy for HCC as well as other cancers.

Conflicts of Interest Statement

The authors have no conflicts of interest.

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References

28. Weaver, J. C. Electroporation of biological membranes from multicellular to nano scales. IEEE Trans on Dielectr Electr Insul 10, 754-768 (2003).
41. Nuccitelli, R., Tran, K., Sheikh, S., Athos, B., Kreis, M., Nuccitelli, P. Optimized nanosecond pulsed electric field therapy can cause murine malignant melanomas to self-destruct with a single treatment. *Int J Cancer* 127, 1727-1736 (2010).

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