33rd Annual International Conference of the IEEE EMBS
Boston, Massachusetts USA, August 30 - September 3, 2011

Nanosecond Pulsed Electric Field Ablation of Hepatocellular Carcinoma

Stephen J. Beebe, Xinhua Chen, Jie A. Liu and Karl H. Schoenbach

Abstract— Hepatocellular carcinoma often evades effective therapy and recurrences are frequent. Recently, nanosecond pulsed electric field (nsPEF) ablation using pulse power technology has emerged as a local-regional, non-thermal, and non-drug therapy for skin cancers. In the studies reported here we use nsPEFs to ablate murine, rat and human HCCs *in vitro* and an ectopic murine Hepa 1-6 HCC *in vivo*. Using pulses with 60 or 300 ns and electric fields as high as 60 kV/cm, murine Hepa 1-6, rat N1S1 and human HepG2 HCC are readily eliminated with changes in caspase-3 activity. Interestingly caspase activities increase in the mouse and human model and decrease in the rat model as electric field strengths are increased. *In vivo*, while sham treated control mice survived an average of 15 days after injection and before humane euthanasia, Hepa 1-6 tumors were eliminated for longer than 50 days with 3 treatments using one hundred pulses with 100 ns at 55 kV/cm. Survival was 40% in mice treated with 30 ns pulses at 55 kV/cm. This study demonstrates that nsPEF ablation is not limited to effectively treating skin cancers and provides a rationale for treating orthotopic hepatocellular carcinoma in pre-clinical applications and ultimately in clinical trials.

INTRODUCTION

The liver performs a wide range of vital functions including roles in metabolism, digestion, protein synthesis, hormone production, glycogen storage, red blood cell destruction and detoxification. Hepatocytes, the major liver cell type, are affected by primary or secondary insults in most forms of liver injury [1]. A basis for cancer-related mutations is continuous hepatocyte turnover due to cell death, which is coupled to apoptosis, necrosis, inflammation and fibrosis [2, 3]. Thus, hepatocyte apoptosis is distinct from apoptosis that occurs during development and immune cell turnover. Long term, chronic pro-apoptotic pressure in the liver promotes developments of apoptosis evasion [4], a well-characterized cancer hallmark [5]. Thus, persistent apoptotic stimuli can be an ultimate harbinger of hepatic cancer, which is the third most common cause of mortality worldwide [6]. The prognosis for hepatocellular carcinoma (HCC) is usually poor because symptoms and diagnoses occur at advanced stages. Although heightened awareness has led to early diagnosis and more successful treatments, incidences of HCC have significantly increased in the U.S. between 2001 and 2006 with an average annual percentage change of 3.5% [7]. Using the Barcelona-Clinic Liver Cancer (BCLC) classification schedule [8], treatments for HCC in very early (stage O), early (Stage A) and intermediate stages (Stage B) include resection and several local therapies including, percutaneous treatments with ethanol or acetic acid, cryotherapy, transcatheter arterial chemoembolization and ablation with radiofrequency (RFA). RFA is now commonly used; however, convincing data to support its value over other local treatments is absent [9]. Furthermore, there are several potential major complications including monitoring the ablation zone, heat-sink effects, pneumothorax, hemoperitoneum, portal thrombosis, capsular hematoma, infections and/or neoplastic seeding [7, 9]. Doxorubicin is the traditional reference standard for evaluating new agents; however, chemotherapeutic agents are usually not effective due to resistances related to changes in expression of target proteins and altered drug metabolism [6]. New treatments are needed for very early, early and intermediate disease stages that avoid recurrences of intrahepatic nodules.

Two new, emerging, non-thermal therapies involve the use of electric fields. Irreversible electroporation (IRE) uses relatively long pulses (milliseconds or microseconds) and low electric fields (V or low kV/cm) [10]. Nanosecond pulsed electric field (nsPEF) ablation using pulse power with much shorter pulse durations (ns) and higher electric fields (high kV/cm) [11, 12]. Pulse power technology instantaneously delivers conditions of high power and low energy. Thus, nsPEF ablation removes tissues by depositing intense, non-thermal, high power electric fields into tumors instead of heat, drugs or chemical agents.

In this study, nsPEF ablation eliminates mouse, rat and human HCC cell types *in vitro* and mouse Hepa 1-6 HCC *in vivo* coincident with apoptosis-like characteristics, a major cancer therapeutic target. NsPEF ablation strategies can be used alone or in combination with other therapeutic agents as a treatment for very early (stage O), early (Stage A) and intermediate stages (Stage B) HCC.

MATERIALS AND METHODS

Cell culture and treatment with nsPEFs: Mouse Hepa 1-6, rat N1S1 and human HepG2 cell lines were obtained from ATTC, cultured as recommended and analyzed in log phase growth. Cells were exposed to one, three or ten pulses with

Manuscript submitted March 26, 2011. This work was supported by the Frank Reidy Research Center for Bioelectrics and the Department of Biology at Old Dominion University, Norfolk, VA.

Stephen J Beebe is with the Frank Reidy Research Center for Bioelectrics, Old Dominion University, Norfolk, VA 23508 USA. (phone: 757-683-2405; fax: 757-451-1010; e-mail: sbeebe@odu.edu).

Xinhua Chen was with Frank Reidy Research Center for Bioelectrics and the Department of Biology at Old Dominion University, Norfolk, VA. She is now in The Department of Hepatobiliary Surgery, the First Affiliated Hospital Zhejiang University School of Medicine, Hangzhou, Zhejiang, China, 310003. (e-mail: xinhuachen_2004@yahoo.com).

Jie A. Liu was with Frank Reidy Research Center for Bioelectrics and the Department of Biology at Old Dominion University, Norfolk, VA 23508, USA. She has now graduated with a MS in Biology

Karl H. Schoenbach is with the Frank Reidy Research Center for Bioelectrics, Old Dominion University, Norfolk, VA 23508 USA. (kschoenb@odu.edu)

60 ns or 300 ns durations at various electric fields in cuvettes with a 0.1 cm gap at 7.7 x 10^6 cells / ml.

Cell viability studies: Two different cell survival methods were used. Control and treated cells (see above) were cultured for 24 hours. In mouse Hepa 1-6 and human HepG2, cells were trypsinized and trypan blue negative cells were counted using a hemocytometer. For rat N1S1, cells were treated with nsPEFs and seeded at 10^5 cells per well in 96-well plates. Then, 24 hours later the ATP content was determined using CellTiter-Glo Luminescent Cell Viability Assay (Promega). Luminescent signals for ATP levels were analyzed with a microplate luminometer (Gemini XPS, Molecular Devices, CA).

Determination of caspase activity *in vitro*: Hepa 1-6 and HepG2 cells were treated by 10 pulses with 60 ns and 300 ns PEFs at 0, 12, 24, 48 and 60 kV/cm and then cultured for 1 hour. Cells treated by camptothecin were used as positive controls. FAM-LETD-FMK caspase-3 FLICA^{TM} kit (Immunochemistry Technologies, LLC) was used to stain caspase positive cells. The cells were analyzed by flow cytometry and expressed as a percentage of the number caspase-3 positive (activated) cell. Results indicate means ± SE (n=3).

Murine tumor model: Animal experiments were approved by the Old Dominion University Institutional Animal Care and Use Committee. Tumors were induced by subcutaneous injection of Hepa 1-6 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 and were treated at this size.

Determination of Caspase-3 activity in Hepa 1-6 tumors treated *ex vivo*: Growing Hepa 1-6 tumors (~0.5 cm) were surgically removed, cut into small fragments, inserted into cuvettes with a 0.1 cm gap as suspensions in PBS and treated with nsPEFs. Two hours after treatment and incubation, control and treated tumor fragments were minced in PBS and homogenized with ground glass homogenizers. The extracts were centrifuged at 15,000xg for 20 minutes and the supernatants were assayed for caspase-3 activity. Catalytic caspase-3 activity was determined using a fluorometric immunosorbent assay (FIENA) for specific and quantitative *in vitro* determination of caspase-3 activity according to the manufacturer's instructions (Roche). An anti-caspase-3 antibody was coated onto 96 well plates. Tumor extracts from control and pulsed electric field-treated tumors were added to the plate and caspase-3 from the extract adsorbed by the antibody. After washing, the caspase-3-selective substrate Ac-DEVD-afc was added. Catalytic activity was determined by fluorescence (Arbitrary Units). As a control, the irreversible inhibitor z-VAD was added to demonstrate specific inhibition of caspase activity.

Survival studies after treatment of Hepa 1-6 tumors *in vivo*: The high voltage pulses were supplied by a Blumlein line pulse generator as previously described and delivered to tumors by a needle array or ring electrode [13]. 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 (0.3-0.5cm). Control tumors were sham-treated and nsPEF tumors were given 30 ns or 100 ns pulses with various electric fields and pulse numbers. Tumors were treated on days 1, 3 and 5. Mice were followed for at least 48 days after treatment except those humanely euthanized with excessive tumor burden (2 cm).

RESULTS

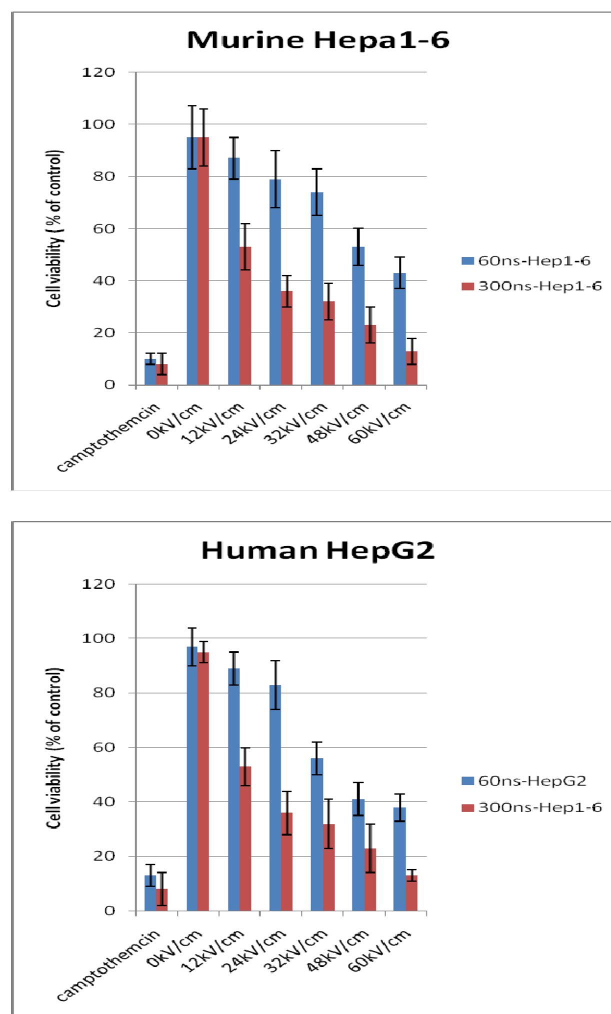

Fig. 1. Effect of nsPEFs on murine HCC cells: Murine Hepa 1-6 (top panel) and human HepG2 cells (bottom panel) were treated with ten pulses at the indicated pulse durations and electric fields as indicated in Methods and Materials. Cell viability was determined 24 hours later by counting cells with a hemocytometer.

NsPEFs decrease survival of murine and human HCC cells: NsPEFs have been shown to decrease viability in several human and murine cell lines [14-16], all of which expressed increases in apoptosis markers that were coincident with decreases in cell viability, suggesting demise, at least in part, by apoptosis. In order to determine whether nsPEFs would decrease survival of HCC cells *in vitro*, murine Hepa 1-6 and human HepG2 HCC cells were treated with ten 60 ns or 300 ns pulses at various electric fields. Cell numbers were determined 24 hours after

treatment (Fig. 1). Cell numbers in both cell lines were decreased in electric field-dependent manners. For a given electric field, 300 ns pulses were more effective than 60 ns pulses. At 60 kV/cm, 300 ns pulses eliminated ~90% of cells while 60 ns pulses only reduced cell numbers by about 60%. The LD50 values for 300 ns pulses (~12 kV/cm) and for 60 ns pulses (32-48 kV/cm) were the same for both cell types.

NsPEFs decrease survival of rat N1S1 HCC cells: Figure 2 shows survival of rat N1S1 HCC cells in response to nsPEFs. N1S1 cells were first exposed to ten pulses at 60 ns or 300 ns at electric fields up to 60 kV/cm. Cell survival was determined 24 hours later by measuring the ATP concentration as a determinant of cell number. The ATP concentration was shown to be directly proportional to cell number as determined in standard curves in each experiment (data not shown). When N1S1 cells were exposed to ten 60 ns pulses, even at the highest electric field, cell survival was decreased by less than 10% (data not shown). For ten 300 ns pulses, cell viability was decreased by as much as 90% with electric fields from 32-60 kV/cm (Fig. 2). Therefore, N1S1 cells were treated with one, three or ten pulses at 300 ns with various electric fields. One and three pulses at 300 ns demonstrated electric field-dependent decreases in cell survival. With single pulses, 60 kV/cm decreased cell numbers to about 40-50% of control cell numbers, representing a near LD50 for these conditions. For 3 pulses an estimated electric field LD50 of between 42 and 60 kV/cm was determined. For ten pulses, about 90% of cells were eliminated with 60 kV/cm and an LD50 value was less than 32 kV/cm.

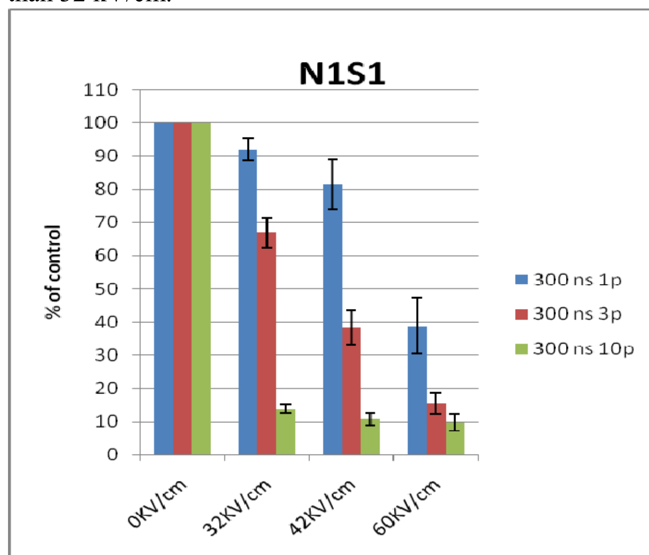

Fig. 2. Effects of nsPEFs on rat N1S1 cells: Rat N1S1 cells were treated with one, three or ten 300 ns pulses at the indicated electric fields as described in Methods and Materials. Cell viability was determined 24 hours later by measuring the ATP concentration as a determinant of viable cell number.

NsPEFs activate caspase-3 in Hepa 1-6 and HepG2 HCC cells *in vitro*: Figure 3 shows caspase-3/7 activity in Hepa 1-6 tumors treated *in vitro* with ten 60 or 300 ns pulses and electric field intensities between 12-60 kV/cm. One hour after treatment, cells were incubated with a cell permeable irreversible inhibitor of caspase-3/7 and analyzed by flow cytometry. As a positive control, cells were incubated with camptothecin for 4 hours. Both mouse and human cells exhibited an electric field-dependent increase in caspase-3/7 positive cells with 30-50% of cells showing the presence of active caspase-3/7 1 hour after treatment. There were smaller increases in active caspases using 60 ns pulses compared to 300 ns pulses at each electric field.

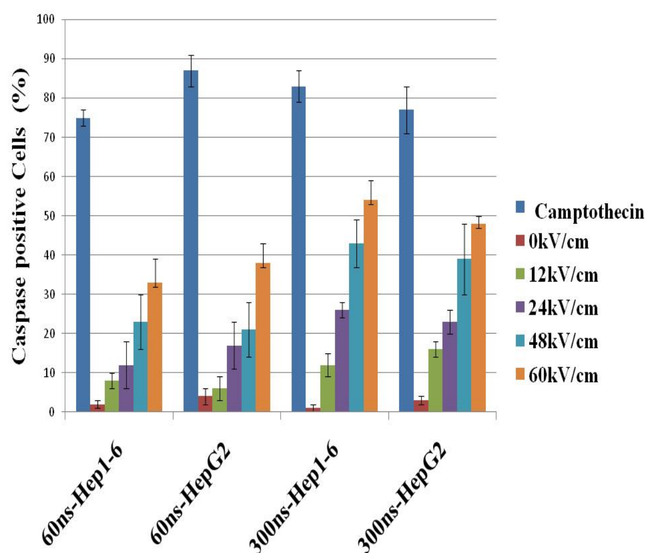

Fig. 3. Hepa 1-6 and HepG2 cells were treated with ten 60 or 300 ns pulses with electric fields between 12 and 60 kV/cm. Cells were assayed for the presence of caspase-3/7 positive cells by flow cytometry 1 hour after treatment as described in Materials and Methods.

NsPEFs activate caspase-3 in Hepa 1-6 tumor tissue treated *ex vivo*. Figure 4 shows caspase-3 activity in Hepa 1-6 tumors treated *ex vivo* with ten 300 ns pulses between 20-45 kV/cm. To identify caspases activity, the assay used a caspase-3-specific antibody and a caspase-3-selective substrate as described in Materials and Methods. Caspase-3 activity increased in an electric field- and time-dependent manner. The greatest activity increases (~5-fold) occurred within the first 30 minutes and maximum activity occurred between 35-45 kV/cm two hours after treatment. Interestingly, in control N1S1 HCC cells, caspase-9 activity, and caspase-3 to a lesser extent, were elevated. These caspase activities decreased with exposure to nsPEFs, a finding that remains to be explained and clearly requires more study (data not shown).

NsPEF ablation of ectopic Hepa 1-6 tumors treated *in vivo* exhibit significant survival times. Figure 5 shows Kaplan-Meier cumulative survival curves for nsPEF treated mice harboring ectopic Hepa 1-6 HCC tumors. The figure shows a subset of larger experimental analyses. Tumors were treated in flanks of mice with 100 pulses at 100 ns or 30 ns with electric fields of 55 kV/cm using needle or ring electrodes on days 1, 3 and 5. Mice were followed for 48-65 days. Untreated mice survived for 12-15 days before tumor burden (2 cm) required euthanasia according to our IACUC protocol. Specific treatment conditions are indicated in the

figure legend. Mice treated with 100ns treatment conditions survived for 48 days before ending the experiment. In another trial, 75% of mice treated with these conditions survived for greater than 250 days (data not shown). For mice treated with 30ns conditions approximately40% of

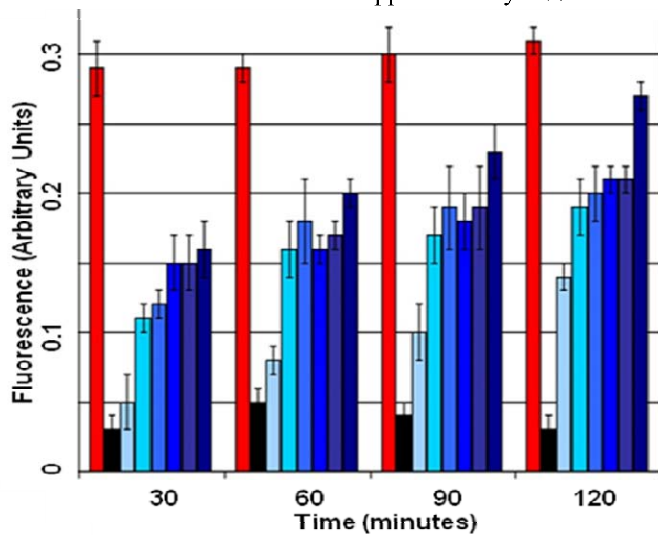

Fig. 4. Effects of nsPEFs on murine Hepa 1-6 tumor tissue caspase activity *ex vivo*: Growing Hepa 1-6 tumors were removed from mice and treated *ex vivo* as described in Materials and Methods. The electric fields were increased from 20 to 45 kV/cm by increments of 5 kV/cm as indicated by darker blue colored bars. The black bars represent untreated controls. During the 2 hours intervals between treatment and caspase assay, tumor fragments were incubated in Hepa 1-6 culture media at 37 degrees C in a cell culture incubator. The data were quantified by fluorescence of cleaved Ac-DEVD-afc substrate by anti-caspase-3 antibody-captured caspases-3.

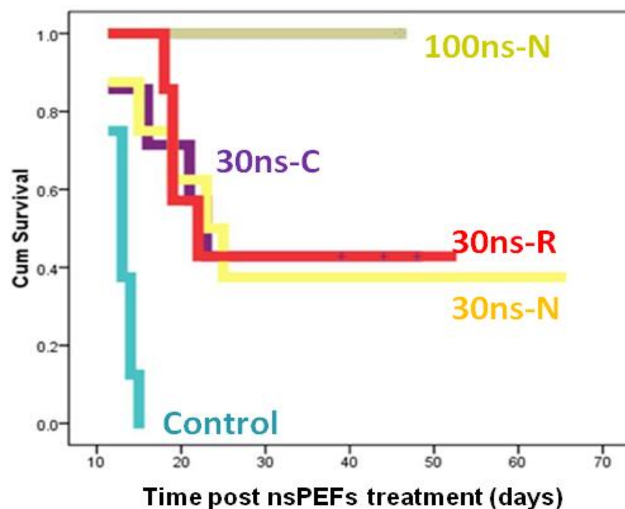

Fig. 5. Effects of nsPEFs on murine Hepa 1-6 tumors treated *in vivo*: Hepa 1-6 tumors were treated *in vivo* as described in Material and Methods. Tumors were treated with 100 pulses at 10 ns or 30 ns durations and electric fields of 55 kV/cm with either a needle array electrode (N) or a ring (R) electrode. One set of mice were treated with a corona effect (30-C, n=7). 100 ns-N (n=24); 30 ns-R (n=7); 30 ns-N (n=8); Control (n=8).

mice survived for as long as 53-65 days before humane euthanasia. For tumor treated with 30ns conditions either with the needle (N) or ring (R) electrode or when coronas (C) occurred, survival was only about 40%.

DISCUSSION

This study demonstrates successful treatment of murine, rat and human HCC *in vitro* and mouse Hepa 1-6 HCC *ex vivo* and *in vivo* using pulse power ablation, a high power, non-thermal, non-drug, local therapy. These results clearly demonstrate that nsPEF ablation is not restricted to treating skin cancers [11, 12]. In all HCC models tested here, signatures of apoptosis were coincident with cell death suggesting that that nsPEFs induces cell death, at least in part, by apoptosis induction. However, other forms of death are likely present and require further analyses.

Hepatocytes are considered to be a major site for most forms of liver injury. Chronic cell turnover provides the basis for apoptosis initiating mutations and over time chronic pro-apoptosis pressure promotes mechanisms to evade apoptosis [4]. Thus, nsPEF reverses a primary initiator of tumorogenesis in HCC. However, apoptotic cell death in tumors where massive cell death occurs without immediate phagocytosis is probably not fully comparable to apoptotic 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, and/or necroptosis. This is highly likely in liver, where apoptotic hepatocyte turnover is coupled to apoptosis, necrosis, inflammation and fibrosis [2-4].

Which HCC stages would be treatable by nsPEF ablation? The studies here and one in normal porcine liver [17] begin to answer this question. It is anticipated that by minimally invasive procedures with laparoscopy, ultrasound guidance and catheter electrodes, nsPEF ablation can substitute for or complement other local ablation treatments. Because essentially all major cellular processes have functional redundancy, targeting a single molecular component of the tumor or its microenvironment does not necessarily suppress HHC progression. Thus, it is reasonabe that therapies targeting HCC tumors or their microenvironment have become more common. Local treatments also have potential to affect the tumor microenvironment. This impacts HCC behavior since the microenvironment dynamically changes as tumors development progresses [18]. The inclusion of nsPEF ablation could provide some therapeutic advantages for HCC patients. Based on data presented here and dependent on successful treatment in an orthotopic HCC model, which is in progress, it is likely that nsPEF ablation could treat the same HCC stages as those stages local, non-resection therapies. These include Stages O (very early), Stage A (early) and Stage B (intermediate) could benefit from nsPEF ablation. NsPEF ablation has significant advantages over other local regional treatments. These include non-thermal ablation, a well-defined treatment zone, no requirement for neuromuscular blockers under optimal conditions and a major therapeutic target for apoptosis induction to override the cancer hallmark of apoptosis evasion.

There are a number of advantages for using nsPEF ablation as a means for cancer therapy as opposed to other physical methods that rely on overt necrosis for tumor cell death. First, nsPEF ablation not only targets programmed

cell death mechanisms for apoptosis induction but also reduces microvascular density markers, suggesting role in anti-angiogenesis. These are two well know cancer hallmarks, the latter necessary for a third cancer hallmark, invasion and metastasis [12]. In this same context, nsPEFs also exhibit tumor-vascular disrupting activity [11, 12, 19], especially with small feeder vessels that are important for immediate oxygenation and nutrition, the absence of which causes local stresses. This represents another attractive therapeutic target [20]. Given these targets, it is highly likely that multiple mechanisms of cell death are operative in dying tumor cells after nsPEF ablation. Another advantage is the rapid onset of apoptosis induction and tumor death [12]. This should reduce the chances for resistances and recurrences, which are common with long acting chemotherapeutic agents, some of which can last for months. It appears that when all cells are exposed to sufficient electric fields, no cells escape death, including rapidly growing tumor cells, slower growing host cells that are collaborating with tumor cells and cancer stem cells, all constituting the tumor mass and microenvironment. Finally, nsPEF ablation has minimal local and systemic side effects. All together, these characteristics make nsPEF ablation a potentially valuable therapy to substitute for or complement other local therapies for HCC.

REFERENCES

[1] S. L. Friedman, “Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver,” *Physiol Rev.,* vol. 88, pp.125-172, Jan. 2008.

[2] T. Takehara, T. Tatsumi, T. Suzuki, E.B. Rucker 3rd, L. Hennighausen, M. Jinushi, et al., “Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses,” *Gastroenterology*, vol. 127, pp. :1189-1197, Oct. 2004.

[3] B.Vick, A. Weber, T. Urbanik, T. Maass, A. Teufel, P.H. Krammer, et al., “Knockout of myeloid cell leukemia-1 induces liver damage and increases apoptosis susceptibility of murine hepatocytes,” *Hepatology*, vol. 49, pp. 627-636, Feb. 2009.

[4] H. Malhi, M.E. Guicciardi, G.J. Gores, “Hepatocyte death: a clear and present danger,” *Physiol Rev*., vol. 90, pp. 1165-1194, Jul. 2010.

[5]. D. Hanahan, R.A. Weinberg, “Hallmarks of cancer: the next generation,” *Cell*, vol. 4, pp. 646-674, Mar. 2011.

[6] C.H. Cha, M.W. Saif, B.H. Yamane, S.M. Weber, “Hepatocellular carcinoma: current management,” *Curr Probl Surg.,* vol. 47, pp. 10-67, Jan. 2010

[7] Center for Disease Control. Morbidity and Mortality Weekly Report, May 7, 2010.

[8] J. M. Llovet, J. Fuster, J. Bruix. “The Barcelona approach: diagnosis, staging, and treatment of hepatocellular carcinoma”, *Liver Transpl*., vol. 10, pp. S115-120, Feb. 2004.

[9] J. M. Llovet, R. Vilana, C. Brú, L. Bianchi, J. M. Salmeron, L. Boix, S. Ganau, M. Sala, M. Pagès, C. Ayuso, M. Solé, J. Rodés, et al., “Increased risk of tumor seeding after percutaneous radiofrequency ablation for single hepatocellular carcinoma”, *Hepatology* vol. 33, pp.1124-1129, May 2001.

[10]. J.F. Edd, L. Horowitz, R.V. Davalos, L.M, Mir, B. Rubinsky, “In vivo results of a new focal tissue ablation,” technique: irreversible electroporation, *IEEE Trans Biomed Eng*., vol 53, pp.1409-1415, Jul. 2006.

[11]. R. Nuccitelli, U. Pliquett, X. Chen, W. Ford, J.R. Swanson, S.J. Beebe, et al., “Nanosecond pulsed electric fields cause melanomas to self-destruct,” *Biochem Biophys Res Commun*., vol. 343, pp. 351-360. May 2006

[12]. X. Chen, J.F. Kolb, R.J. Swanson, K.H. Schoenbach, S.J. Beebe, “Apoptosis initiation and angiogenesis inhibition: melanoma targets for nanosecond pulsed electric fields,” *Pigment Cell Melanoma Res*., vol. 23, pp. 554-563, Aug. 2010.

[13]. J.F. Kolb, X. Chen, J. Zhuang, W. Ren, N. Scully, R.J. Swanson, S.J. Beebe, K.H. Schoenbach, “Tumor treatment with nanosecond pulsed electric fields,” *IEEE Pulse Power Conference* 2009; pg.880-885.

[14]. E.H. Hall, K.H. Schoenbach, S.J. Beebe, “Nanosecond pulsed electric fields induce apoptosis in p53-wildtype and p53-null HCT116 colon carcinoma cells,” *Apoptosis*, vol. 12, pp.1721-1731, Sept. 2007.

[15] W.E. Ford, W. Ren, P.F. Blackmore, K.H. Schoenbach, S.J. Beebe, “Nanosecond pulsed electric fields stimulate apoptosis without release of pro-apoptotic factors from mitochondria in B16f10 melanoma,” *Arch Biochem Biophys*., vol. 497, pp. 82-89 May. 2010

[16] W. Ren, S.J. Beebe, “An apoptosis targeted stimulus with nanosecond pulsed electric fields (nsPEFs) in E4 squamous cell carcinoma,” *Apoptosis*, in press. Jan. 2011.

[17]. G. Long, P. Shires, D. Plescia, S Beebe, J. Kolb, K. H. Schoenbach, “Targeted tissue ablation with nanosecond pulses,” *IEEE Transactions on Biomedical Engineering*, in press.

[18] J.D, Yang, I. Nakamura, L.R. Roberts, “The tumor microenvironment in hepatocellular carcinoma: Current status and therapeutic targets”, *Semin Cancer Biol*., vol. 21, pp. 35-43, Feb. 2011.

[19] X. Chen, R. J. Swanson, J. F. Kolb, R. Nuccitelli, K. H. Schoenbach, “Histopathology of normal skin and melanomas after nanosecond pulsed electric field treatment”, *Melanoma Res*., vol. 19, pp. 361-371, Aug. 2009.

[20] M. J. McKeage, B. C. Baguley, “Disrupting established tumor blood vessels: an emerging therapeutic strategy for cancer”, Cancer., vol. 116, pp. 1859-1871, Apr. 2010.