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Optimized Nanosecond Pulsed Electric Field Therapy Can Cause Murine Malignant Melanomas to Self-Destruct with a Single Treatment

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Abstract

We have identified a new, nanosecond pulsed electric field (nsPEF) therapy capable of eliminating murine melanomas located in the skin with a single treatment. When these optimized parameters are used, nsPEFs initiate apoptosis without hyperthermia. We have developed new suction electrodes that are compatible with human skin and have applied them to a xenograft nude mouse melanoma model system to identify the optimal field strength, pulse frequency and pulse number for the treatment of murine melanomas. A single treatment using the optimal pulse parameters (2000 pulses, 100 ns in duration, 30 kV/cm in amplitude at a pulse frequency of 5–7 pulses/s) eliminated all 17 melanomas treated with those parameters in 4 mice. This was the highest pulse frequency that we could use without raising the treated skin tumor temperature above 40 °C. We also demonstrate that the effects of nsPEF therapy are highly localized to only cells located between electrodes and results in very little scarring of the nsPEF-treated skin.

Keywords

nanosecond; pulsed electric fields; apoptosis; necrosis; skin cancer

The most common treatment for skin cancer is the surgical removal of the lesion. This is time consuming and almost always leaves a scar. An alternative approach is electrochemotherapy in which the tumor is exposed to a toxic drug after electropermeabilization using electric pulses in the microsecond domain1. The approach we use here applies electric pulses in the nanosecond domain. In 2002, Beebe et al.2 first showed that applying ultrashort, nanosecond pu lsed electric fields (nsPEF) to mammalian cells and solid tumors results in reduced tumor growth, and induction of apoptosis in the treated cells. Subsequent research on nsPEF over the past 8 years has shown that, unlike thermal ablation which causes a wide area of tissue necrosis, nsPEF is unique in its mechanism of action which is predominantly non-thermal and subcellular, inducing apoptosis via intracellular membrane changes3;4. In addition, nsPEF application disrupts tumor blood flow and only affects tissues localized between the delivery electrodes5;6. As we demonstrate in this study, tissues just outside the edge of the suction electrode show no effects from the therapy.

We recently completed a longer term study in which 17 SKH-1 immune-competent mice with a single melanoma received one to three treatments with nsPEF as needed to eliminate the tumor7. Tumors exhibited complete remission in every case and did not recur for at least

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4 months at which time the animals were euthanized. One drawback of using SKH-1 mice is that they have a strong immune system that can slow or even halt tumor growth. Therefore in all of the work described here we have used athymic nude (Nu/Nu) mice in which melanomas grow continuously and more rapidly. These tumors develop from the cultured murine melanoma cells injected into the subcutaneous compartment of these mice rather than developing from the mouse's own tissues. However, these tumors exhibit both angiogenesis and metastasis and this will eventually kill the mouse if not treated.

Additional innovations introduced here include a new triggered spark gap pulse generator, a shorter (100 ns) pulse length, and a new suction electrode pulse delivery system compatible with human skin. We have methodically varied the nsPEF parameters of pulse number, amplitude and frequency in order to determine the optimal therapy for treating murine melanoma with these suction electrodes.

Materials and Methods

Cell lines

Murine B16-F10 melanoma cells transfected with enhanced green fluorescent protein (eGFP) were obtained from Dr. Alan Houghton at the Memorial Sloan-Kettering Cancer Center on 11/23/2008 and stored in liquid nitrogen until use. For authentication of this cell line we labeled our cells in culture with antibodies to the melanoma antigen, gp100 (Santa Cruz Biotechnology sc-33590, Santa Cruz, CA) and used a goat anti-rabbit secondary from Invitrogen (Carlsbad, CA). We confirmed detection of gp100 on these cells while no labeling was observed in negative controls for which only the secondary antibody was added.

Frozen vials were thawed and cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 200 mM L-Glutamine (Cellgro, Mediatech, Herndon, VA), and 2% Penicillin-Streptomycin (Mediatech). Cultures were maintained in a 5% CO_2 incubator at 37°C.

Animals

4–6 week old female Nu/Nu mice (immunodeficient, hairless, albino) from Charles River (Boston MA) were housed at 22°C for at least one week prior to use. Mice were under isoflurane anesthesia and positioned on a 37 °C warming bed for tumor cell injections, photography and nsPEF treatments. All procedures were approved by the IACUC of In-Vivo Technologies (Burlingame, CA).

Melanoma Induction

Melanomas were formed by injecting 10^6 B16-F10-eGFP cells in 15 μl of HBSS (Hank's Buffered Salt Solution) under the skin using a hypodermic syringe while the mice were under 1.6% isoflurane inhalation anesthesia. Each mouse had a maximum of four injection sites. Tumors were detected visually by the bulges they produced and by GFP detection under fluorescent microscopy. Typically, tumors grew to 3–4 mm in diameter by day five, post-injection. Daily photographs were taken by surface view, transillumination, and fluorescent imaging using a Leica (Bannockburn, USA) MZ16F stereoscope before and after nsPEF treatment.

Suction Electrodes

We constructed three different suction electrodes (fig. 1A) and tested four different electrode configurations. All of the suction electrodes are plastic cylinders with a cup-shaped opening at one end with an inner diameter of 4 mm and a depth of 2 mm. The distance between

opposing electrodes when they are on the sides of the cup-shaped opening is 4 mm. For the needle electrode, the distance between the center needle and each of the four outer needles is 2 mm. The base of each cup has small holes drilled in it to allow a suction to be applied from the opposite end of the cylinder to pull the tumor into the cavity. The metal electrodes were positioned either as flat or cylindrical pieces in the walls of the cavity or as needles protruding from the base. This design restricts nsPEF application to only those tissues located between electrodes within the suction cup. We demonstrated that this design was compatible with human skin in several regions of the body (fig. 1B).

The construction process began with 316 stainless steel tube or rod. After the appropriate cuts were made the electrodes were electropolished in a 2:1 solution of concentrated phosphoric:sulfuric acid. Electropolishing reduces undesirable corona discharge by removing sharp edges that focus and enhance the electric field. For this process the acid was maintained at 85°C with constant stirring. The positive electropolishing electrode was a hollow copper cylinder, concentric with the steel electrode. A sufficient negative voltage (≈ − 4 v) was applied to the steel electrode to maintain a current of 8 amps. After 5 min of current flow the electrode was removed from the acid solution and placed in a saturated sodium metabisulfite solution for one minute. The electrode cup was formed by placing a cylinder-shaped plug of Alumilite mold putty between the electrode faces to exclude resin there and Palapress Vario resin (CE0197, Heraeus Kulzer Co., Hanau Germany) was poured into a mold holding the electrodes in position. This resulted in the electrodes being embedded in the plastic with only one side exposed along the inside wall of the suction cup.

100 Nanosecond Pulse Generator

The nsPEF generator utilizes a Blumlein line to create fixed, short pulses of high voltage with 15 ns rise and fall times (fig. 1G). It works by charging up a coaxial cable (through a current limiting resistor) to a high voltage, creating a capacitor on the coaxial cable between the inner conductor and its outer conductive shielding. When the inner conductor is rapidly brought to ground by a switch, a corresponding pulse is generated across the outer shielding. The duration of this pulse is determined by the length of cable used, and the amplitude is determined by the voltage at which the coaxial cable was charged. By this method, it is possible to precisely control the number of pulses delivered as well as the frequency of those pulses simply by controlling the discharge switch for the coaxial cable's inner conductor. The generator itself (including all components) fits into a rolling suitcase measuring 24" × 15" × 18" (fig. 1C–E)

Microcontroller—The system we designed uses a Microchip PIC16F887 microcontroller to handle all the necessary aspects of controlled pulse delivery. The controller's circuit board is housed in an external metal box (fig. 1F), and all inputs and outputs to the rest of the system are implemented with fiber optics to create isolation. The user is able to feed information to the microcontroller-based system via keypad, and a liquid crystal display (LCD) on the box delivers important information to the user. The circuit is battery-powered, using a regulated single supply voltage rail of +5VDC. When the user wants to begin a treatment of pulses at a particular voltage and frequency, the microcontroller is responsible for enabling the high-voltage supply, setting the voltage for that supply's output, and sending the precisely-timed signal to the switch which is used to discharge the coaxial cable.

High Voltage Generator—The high-voltage power supply we use in the Blumlein line pulse generator is a Matsusada RB30-30P. It can output 0–30kV @ 1mA, and requires a +24VDC power supply capable of sourcing 2A of current. It has a control pin which enables/disables the output voltage. There is also an analog input pin (0–5VDC) which is scaled by the Matsusada supply to generate its 0–30kV output. For the microcontroller to

communicate with the RB30-30P, a receiving board has been created which reads the fiber-optic cables from the control box and translates them to the electrical signals that the power supply requires.

Switch—The switch we use to rapidly discharge the coaxial cable is a spark gap at atmospheric pressure. This type of switch meets our requirements for the Blumlein system in that it is extremely fast, it can accommodate very high voltages, and it can also be easily controlled. The way we control this spark gap switch is with another, smaller spark gap. The switch system is thus a two-part switch.

We use a coaxial cable length of 20 meters in order to generate a pulse duration of 100 ns. This cable length made possible a more compact and portable pulse generator design than our previous 300 ns pulser. However, in order to deliver the same energy to the tumor, this reduction in pulse duration by a factor of 3 required that we increase the number of pulses by at least a factor of 3. Our previous therapy of 300 pulses suggested that a minimum of 900 pulses would be required so we examined a range of 500–2700 pulses.

nsPEF Treatment

Tumors were first coated with olive oil before sucking them into the electrode cup to reduce the occurrence of air pockets between the electrodes. The low dielectric constant of air can lead to ionization of the gas and current flow in the form of a spark passing over the skin and bypassing the tumor. We used a hand vacuum pump (MityVac, model MMXA, St. Louis, MO) to pull the tumors up into the electrode's suction cup with a suction of 500 mm Hg. Treatment was paused if sparking occurred between electrodes and additional olive oil was applied before again pulling the tumor into the suction cup. The suction electrode was rotated 45° every 500 pulses to ensure a uniform field distribution across the tumor.

All pulses applied were 100 ns long and we varied either the pulse number, amplitude or frequency. The current delivered to the tumor was measured using a Pearson coil surrounding the lead wire connected to the delivery electrode. Tumors that showed signs of regrowth, generally indicated by renewed GFP production, were retreated using the same parameters as the first treatment. Tumors were considered eliminated when no regrowth was detected within 2 weeks after the last treatment.

Histology

Nu/Nu mice have no T cells and tumors grow rapidly. If any tumor cells are not triggered to undergo apoptosis following nsPEF treatment, tumor recurrence is readily visible by GFP fluorescence within 10 days. Mice showing complete tumor regression for 2–4 weeks (mean of 22 days) were euthanized and skin samples were fixed for histology. The skin from each tumor location was cut out and immediately placed in a 10% buffered formalin solution for at least 24 hours at room temperature. After paraffin embedding, 5 μm sections were cut every 100 μm across the entire skin sample and stained with H&E. Stained slides were observed using microscopy for signs of melanoma cells. Photographs from each slide were captured with a camera mounted on a light microscope.

Measuring Tumor Temperature

We measured the temperature in the tumor during nsPEF treatment using a 75 μm-wide thermocouple (model 5TC-TT-K-40-36, Omega Engineering, Stamford, CT) with a reference ice bath. The thermocouple is inserted into the tumor by first making a small hole through the skin and into the tumor using a 20 gauge hypodermic needle. We then insert the thermocouple into this opening just before sucking the tumor into the suction electrode. The thermocouple is optically isolated from ground using a battery-powered voltage-to-

frequency converter that drives an LED signal carried over a fiber optic line. On the receiving end of the fiber optic, we convert the light frequency back to voltage which is fed into an Agilent 34405A digital multimeter with a frequency function. Agilent software allows us to datalog this frequency at regular intervals. The thermocouple temperature is derived from a calibration curve generated earlier using a heated water bath.

Results

Optimizing Suction Electrode Configuration

Our previous delivery device for applying nsPEF to murine melanomas sandwiched a fold of skin containing a melanoma between parallel plate electrodes. That approach is not compatible with many regions of human skin that cannot be lifted away from the body as easily as mouse skin. Consequently, we developed a new design compatible with human skin that pulls skin up into a shallow cup using suction. We tested four different electrode configurations within the suction cup to treat GFP-expressing B16-F10 melanoma tumors on the backs of nude mice (fig. 1A & 2A). Controls included applying suction without nsPEF for the same 10 min treatment period as that used for nsPEF application, as well as applying suction while heating the suction cup and tumor by 7 °C, the maximum temperature increase measured during nsPEF application. Neither of the control treatments resulted in a reduction in GFP fluorescence. In contrast, nsPEF treatment of these tumors (2700 pulses, 100 ns, 30 kV/cm, 5–7 Hz) resulted in the rapid reduction in GFP fluorescence and the rate of fluorescence reduction correlated well with the longer term remission of the melanoma (fig. 2B, 3B). All of the suction electrode configurations delivered nsPEF that reduced GFP production, but the 6-pole dual electrode configuration reduced the GFP signal faster than the others. In most cases, GFP could no longer be detected 1 day after treatment. When we then studied the rate of tumor shrinkage following nsPEF treatment using the four different electrode configurations, we found that the 6-pole dual configuration again resulted in the fastest tumor shrinkage rate (fig. 3B). Since this electrode configuration appeared to be the most effective at triggering tumor remission, we decided to conduct all of the following studies to optimize nsPEF pulse parameters using this electrode.

NsPEF Effects Are Highly Localized

We studied the early morphological changes in nsPEF-treated tumors and skin as well as skin immediately adjacent to the suction electrode cup in H&E stained paraffin sections (fig. 4). We detected no changes in the epidermis or capillaries in the adjacent regions, but observed striking changes in the treated tissue. Within one hour after nsPEF application, narrow capillaries were no longer visible. Blood cells appeared to flow in wide “streams” unbounded by capillaries throughout the tumor and many fused together by 48 h. Both epidermal and melanoma cells shrank within an hour and the melanoma cell pyknosis seemed to peak by 24 h.

Optimizing Pulse Number

We investigated a wide range of pulse numbers from 500 to 2700 while using the same amplitude, duration and frequency (30 kV/cm, 100 ns, 5 Hz). The smallest pulse number that resulted in the complete remission of tumors 100% of the time is 2000 (Fig. 5A). Using fewer pulses sometimes eliminated the tumor with one treatment but often required more treatments. Treated tumors appeared the same in both coloration and scab area across the entire range of pulse numbers.

Optimizing Pulse Amplitude

We investigated a range of pulse amplitudes using the same pulse duration, number and frequency (100 ns, N=2000, 7 Hz). Field strengths lower than 30 kV/cm did not trigger complete tumor remission with a single treatment, but could sometimes do it with multiple treatments (fig. 5B). The current delivered to the tumor was dependent on field strength, as expected. The 6-pole dual electrode typically delivered 5 amps at 10 kV/cm and 20 amps at 30 kV/cm. This non-linear relationship between voltage and current is most likely due to uneven electropermeabilization of the stratum corneum by our suction electrodes. 10 and 15 kV/cm treatments consistently failed to completely eliminate GFP with a single treatment, resulting in tumors that regrew a few days post treatment. Additional treatments at the same low amplitudes never completely eliminated the tumor. We conclude that the minimum amplitude for triggering complete remission with one treatment is 30 kV/cm.

Optimizing Frequency

In order to minimize the treatment time, it is desirable to use the highest pulse application frequency possible. The main limitation to increasing this frequency is the consequent temperature increase in the tissue. Therefore we introduced a miniature thermocouple into the tumor during pulsing for simultaneous measurement of tumor temperature. We conducted experiments on 10 tumors using frequencies from 1 to 10 Hz. Applying nsPEF (30 kV/cm, 20A) at 1 Hz increased tumor temperature by 2°C, whereas 5 pulses per second increased the tumor temperature by 6–7°C (fig. 6B). Initial skin temperature ranged from 25 to 30 °C because the skin is pulled away from the body during pulsing. We decided to use a maximum frequency of 7 Hz so the tumor temperature would never exceed 37 °C. This is well below the hyperthermia threshold of 41 °C.

Having established 7 Hz as our maximum frequency, we tested the efficacy of various frequencies up to 7 Hz with parameters of 100 ns duration, 30 kV/cm and 2000 pulses (fig. 5C). We found that for 30 kV/cm, applying pulses at 5 Hz or higher was more effective than using lower frequencies. At 1 Hz and 3 Hz, 30% of the tumors began growing again 1– 2 weeks after the initial treatment.

Surface view photographs of treated tumors provide some insight into the required field strength (fig. 6A). Amplitudes of 10 kV/cm cause no scab formation and the tumor growth rate is similar to controls. 15 kV/cm amplitudes produce some scab formation in a circular pattern where edges of the suction electrode contacted the skin. This circular pattern is more pronounced for 20 kV/cm, suggesting that the electric field is affecting more of the skin cells but not all of the skin between the electrodes. When the field strength is increased to 25 kV/cm, the ring-shaped scab is no longer evident, indicating that most skin cells between the electrodes are responding at this field strength. The GFP fluorescence images (fig. 6A) indicate that regrowth occurs in tumors treated with 10–20 kV/cm within 6–8 days after treatment.

Optimal Parameters

From the above results we conclude that the minimum nsPEF parameters required to obtain complete tumor remission with a single treatment using 100 ns pulses are: 2000 pulses, 30 kV/cm, 5–7 Hz, 20 A or greater. Electrodes at the skin surface must first permeabilize the stratum corneum so that current flow can deliver the electric field to the tumor. We find that if this current flow falls below 20 A, the electric field at the tumor is insufficient to trigger apoptosis in all of the tumor cells. Using these pulse parameters, we treated 8 tumors at 5 Hz and 9 tumors at 7 Hz. All of these tumors showed complete regression with a single treatment. In order to confirm complete regression, we conducted histological analysis of the treated regions by preparing a 5 μm section at 0.5 mm intervals throughout the region. Each

section was carefully studied and photographed. No melanoma cells were detected in any of these sections.

Skin Appearance After nsPEF Treatment

Within a few days after nsPEF treatment a hard scab typically forms at the treatment region. The scab falls off by the end of the second week, leaving behind freshly regenerated skin that usually has a different appearance than the surrounding skin (fig. 6C). Over time this coloration difference fades and does not leave a permanent scar.

Discussion

These results bring us closer to our ultimate goal of applying nsPEF technology to treat tumors in humans. We now have a pulse delivery device compatible with human skin and have found a nsPEF therapy that triggers complete regression of murine malignant melanomas with a single 6 minute-long treatment. We have found that 30 kV/cm delivering at least 20 A appears to be the lowest field strength and current that can be used to obtain complete remission with a single treatment. Pulse application is more effective at 5–7 Hz than it is at lower frequencies. One advantage of nsPEF therapy is that only the tissue within the suction cup is exposed to the field, minimizing damage to the healthy tissue surrounding a tumor. Figure 4 illustrates the normal appearance of tissue samples collected from the perimeter of a treated area. We also found that by limiting the 100 ns pulse frequency to 7 Hz or less, we could avoid heating the tumor to hyperthermia temperatures that could damage surrounding tissues.

Our results are consistent with our previous work with 300 ns pulses and parallel plate electrodes[5;7]. Those studies concluded that at least 300 pulses at 40 kV/cm and 0.5 Hz were needed to cause tumor regression and often a second or third treatment was required. Assuming that the critical parameter is the energy delivered to the tumor, by reducing the pulse length to one-third of 300 ns and reducing the pulse amplitude to three-fourths of our previous levels, one would expect that each pulse would deliver 3/12 or ¼ of the energy. Therefore, we would predict that four times more pulses would be required using a 100 ns pulse width. In fact, 1200 pulses did trigger complete regression in 43% of the cases, but we found it necessary to use 2000 pulses to cause complete regression in 100% of the cases.

The critical question remains: how exactly does nsPEF induce apoptosis? The main effect of large electric fields on cells is to permeabilize membranes by driving water into the lipid bilayer. This effect requires about 1 volt per membrane. Thus if the plasma membrane of a 10 μm wide cell were the target, only 2 kV/cm would be sufficient to generate the 1 volt per membrane needed to permeabilize. Our data indicate that a much larger field strength of 30 kV/cm is required for the optimum effect. This suggests that the target membrane is located in a small organelle which would require the larger field strength to generate a voltage gradient of 1 volt across each of the two membrane regions perpendicular to the field lines. Any organelle that spans a distance of 0.7 μm or more would be a good candidate. This target size would encompass many different organelles including the mitochondria and ER. Nanopores in these organelles would release Ca^{2+} into the cytoplasm as has been observed previously [7–9] and that Ca^{2+} increase might initiate the apoptosis pathway. The induction of apoptosis by various drugs or toxins in a wide variety of cancer cells has been found to require similar increases in intracellular Ca^{2+} [10–13]. These increases are typically smaller (< 1 μM) than those associated with necrosis (1–2 μM)[14;15] and similar small changes in $[Ca^{2+}]$ are triggered by nsPEF[7].

Apoptosis occurs normally in many tissues and cell types at the end of their useful life. Most characteristics of apoptosis have been found to be initiated by nsPEF treatment, including

maintenance of plasma membrane integrity16, phosphatidylserine (PS) externalization on the plasma membrane17–20, cytochrome C release from the mitochondria21, caspase activation2;21–25, chromosomal condensation or pyknosis5 and DNA fragmentation7;26 . The first reports of nsPEF-triggered apoptosis came from studies in Jurkat and HL-60 cells. The apoptosis response of these cells in culture depends on the pulse amplitude, duration and number. Shorter pulses in the 10 ns range resulted in slower apoptosis progression. Longer pulses in the 100–300 ns range generally stimulated more rapid apoptosis progression25.

There are certainly other organelles in addition to the ER that are permeabilized by nsPEF27;28. Much more research is needed to determine if permeabilization of other organelles plays a role in triggering tumor remission.

One major advantage of nsPEF therapy for tumor treatment is that it is not cell-specific. Field strengths of 30 kV/cm will generate nanopores in organelle membranes independent of cell type29–33. That means that we should be able to trigger apoptosis in a wide variety of tumor types. Indeed there is already one report of the successful treatment of a human basal cell carcinoma tumor34. Our main challenge is to develop the appropriate delivery devices and pulse parameters for treating tumors with nsPEF. We chose melanomas as our first target because they often appear on the skin which is readily accessible to nsPEF electrode placement. Suction electrodes introduced here have proven to be very effective. Tumors growing within the body will be much more challenging. However, modern imaging techniques such as ultrasound and CAT scan could facilitate placement of electrode arrays around internal tumors, expanding the application of nsPEF therapy beyond skin cancer.

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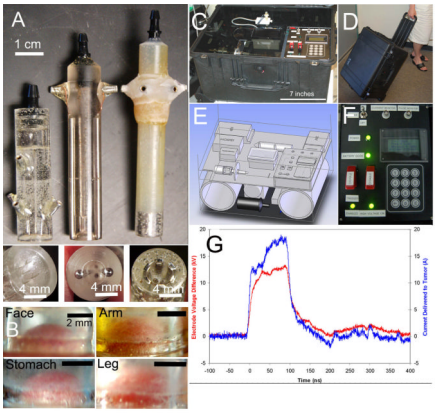

Figure 1.

Suction electrodes and PulseCure System. A. Side view of each electrode along with an end view just beneath each one. The female connectors on the sides provide a direct connection to each electrode. B, Compatibility with human skin demonstrated by photos of different regions of human skin being sucked into a clear plastic suction cup with the same dimensions as these electrodes. C. PulseCure model SS-1 Pulse Generator. Photograph of unit ready for use to treat a mouse tumor. D. PulseCure System is built within a plastic roller case for easy transport. E. 3D model of the components housed in the plastic roller case. The coaxial cable is wound along the inside wall of the cardboard cylinders on the bottom. F. Close-up of control box. G. Pulse voltage and current waveforms from oscilloscope screen when treating mouse skin with a 6-pole dual suction electrode. Rise time of pulse: 15 ns.

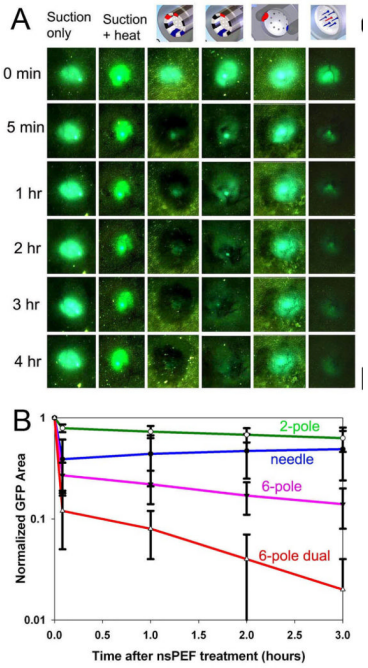

Figure 2.

Typical changes in GFP fluorescence from melanomas in response to nsPEF application (2700 pulses, 100 ns long, 30 kV/cm at a frequency of 5–7 Hz) using the suction electrode configuration indicated above each column **A**. GFP fluorescence changes following nsPEF application using the electrode pictured at the top of each column. The voltage difference was applied between the red and blue electrodes in each case. The 6-pole dual electrode is pictured above the third column from the left followed on the right by the 6-pole single, two pole and the needle configuration. **B**. Normalized change in GFP signal area on a log scale following nsPEF treatment with the indicated suction electrode type. Error bars represent SEM, N=10–12.

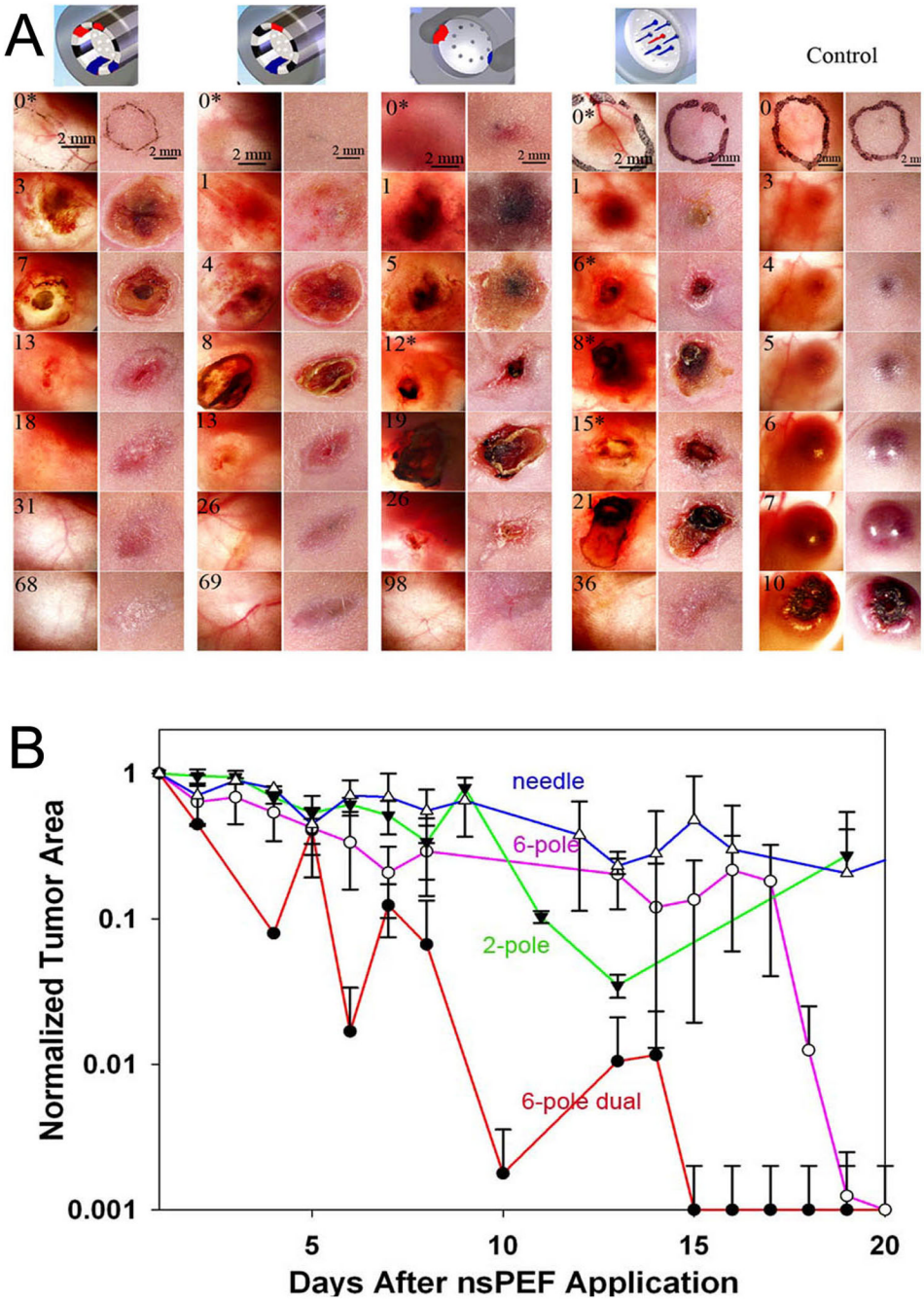

Figure 3.

Typical changes in melanoma over time following a single treatment with nsPEF. **A**. Transillumination and reflected light images of melanomas treated on day 0. Each pair of images were taken on the day after treatment indicated in the upper left corner. An asterisk indicates the days nsPEF treatment occurred. The control tumor was sucked into a dummy electrode and heated to 37 °C for 10 min but no field was applied. By day 10 it began to ulcerate so we had to euthanize the mouse. **B**. Normalized change in tumor area on a log scale following nsPEF treatment with the indicated suction electrode type. Error bars represent SEM, N=10–12.

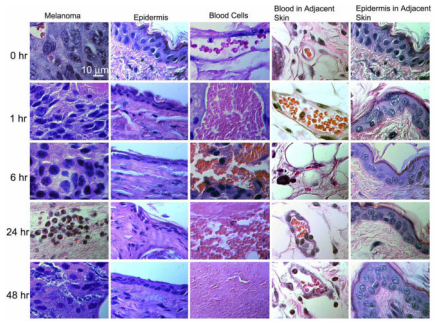

Figure 4.

NsPEF effects on skin and melanomas are highly localized. Histological sections of treated tumors and skin immediately adjacent to suction electrode location at different times after treatment with 2000 pulses, 30 kV/cm at 7 Hz. Scale bar in upper left applies to all images. Images in the three left columns were taken from skin with tumors before (0 hr) and three times after nsPEF treatment. Images in the two right columns were taken from skin immediately adjacent to the nsPEF-treated region and no morphological changes could be detected there.

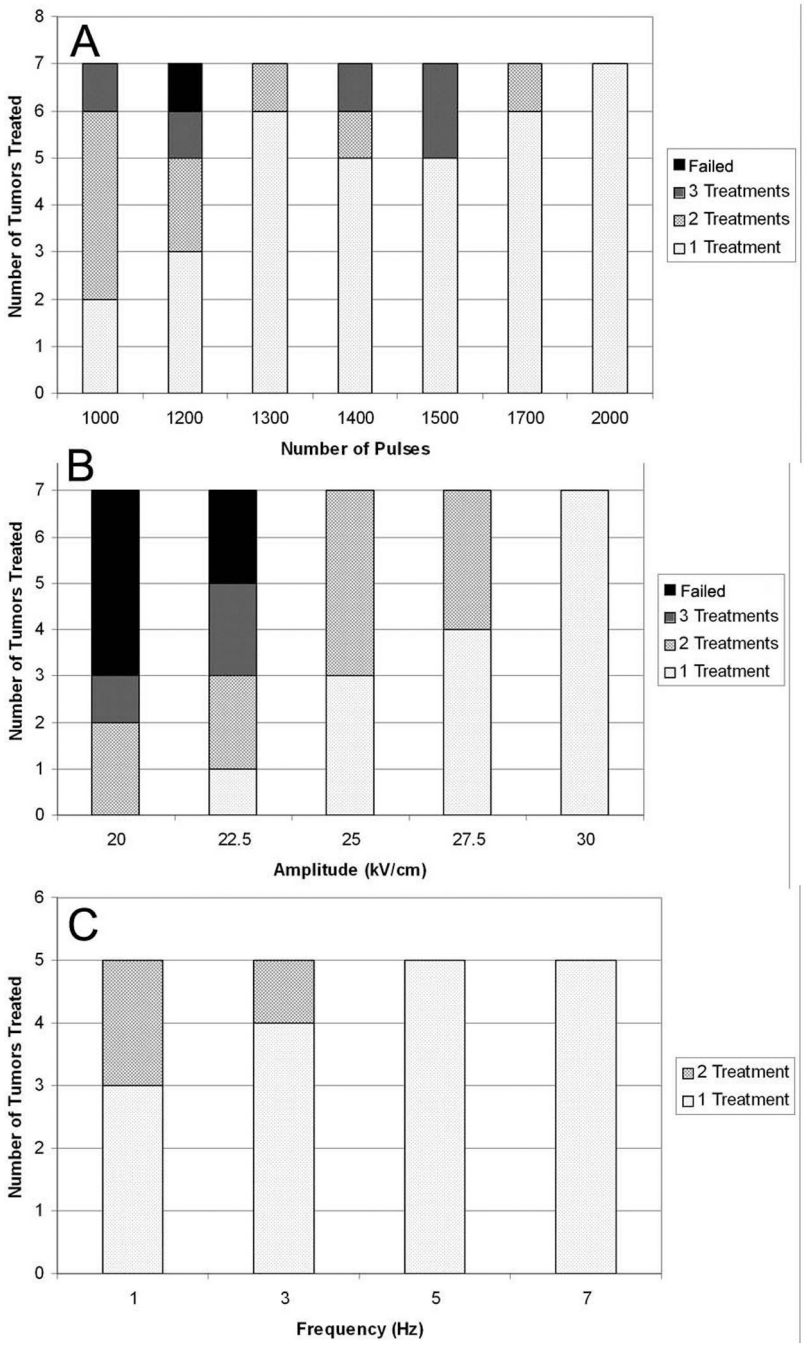

Figure 5.

Pulse parameter optimization data. A. Number of tumors treated with the indicated pulse number and the number of treatments required for complete remission. All treatments used the 6-pole dual electrode, 100 ns duration, and 30 kV/cm at 7 Hz. “Failed” indicates the number of tumors that failed to undergo complete remission following multiple treatments. These typically outgrew the electrode suction cup or developed a scab that made them difficult to treat again. B. Number of tumors treated at the indicated electric field strength along with the number of treatments required to eliminate the tumor. Each treatment used 2000 pulses applied at 7 pulses per second. C. Number of tumors treated at each frequency with the 6-pole dual electrode using 2000 pulses 100 ns long 30 kV/cm.

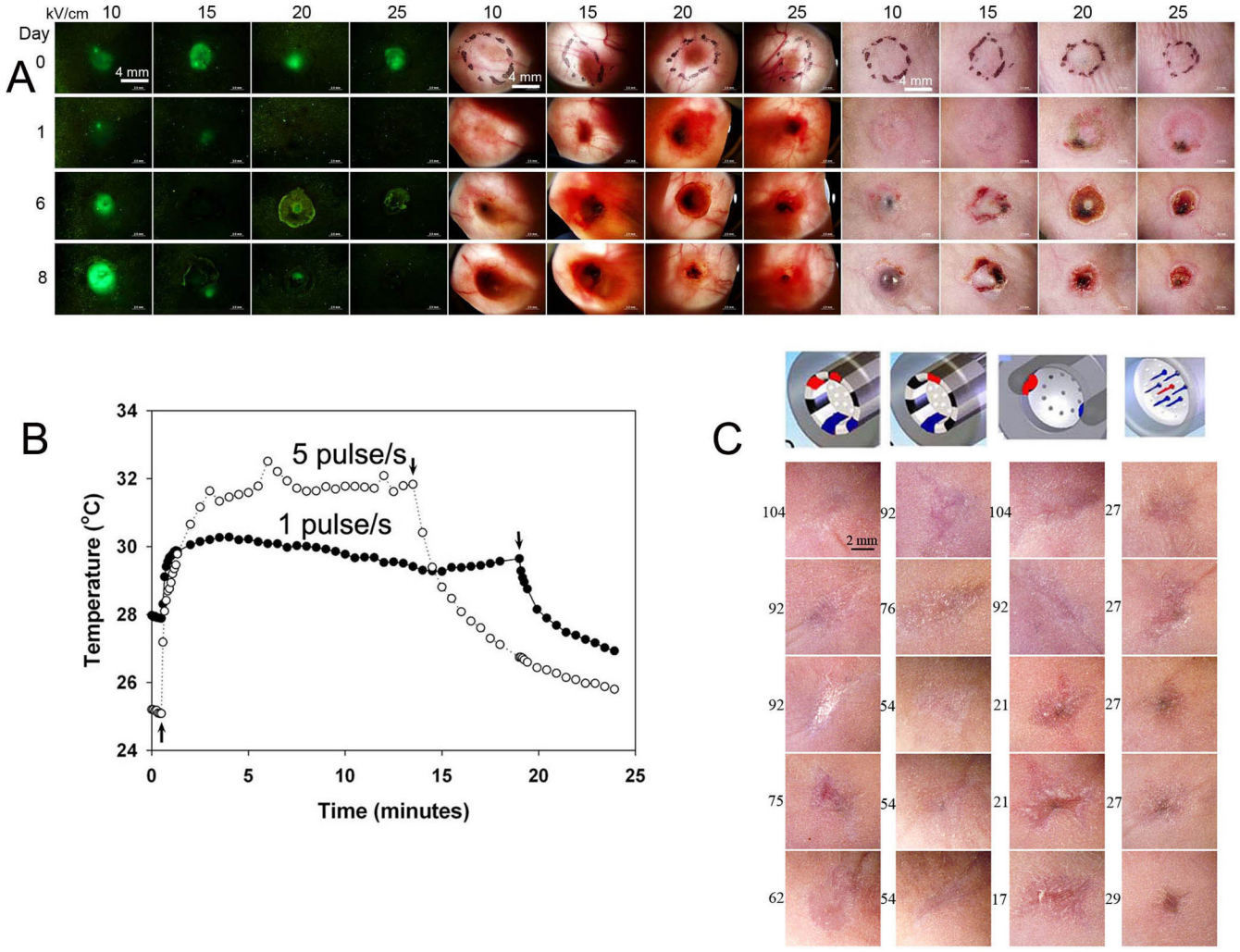

Figure 6.

Typical melanoma responses to nsPEF therapy in the 10–25 kV/cm range. A. Four melanomas on one mouse were treated with either 10, 15, 20 or 25 kV/cm nsPEF (2000 pulses, 100 ns, 7 Hz). GFP fluorescence, transillumination and reflected light images collected over an 8-day period following treatment are presented. B. Temperature increase inside a tumor during nsPEF application. 30 kV/cm pulses 100 ns long were applied beginning at the upward arrow at the indicated frequency. Pulsing was stopped at the downward arrow. 1 Hz pulse application increased tumor temperature by 2 °C and 5 Hz increased the temperature by 7 °C. C. Appearance of nsPEF-treated skin on the indicated day following nsPEF therapy. Reflected light images of 20 different nsPEF-treated tumors taken on the day after treatment indicated to the left of each photo. The electrode configuration used is at the top of each column.