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Non-thermal Nanoelectroablation of UV-induced Murine Melanomas Stimulates an Immune Response

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Summary

Non-thermal nanoelectroablation therapy completely ablates UV-induced murine melanomas. C57/BL6-HGF/SF transgenic mice were exposed to UV radiation as pups and began to develop visible melanomas 5–6 months later. We have treated 27 of these melanomas in 14 mice with nanosecond pulsed electric field (nsPEF) therapy delivering 2000 electric pulses each 100 ns long and 30 kV/cm at a rate of 5–7 pulses per second. All nanoelectroablated melanoma tumors began to shrink within a day after treatment and gradually disappeared over a period of 12–29 days. Pyknosis of nuclei was evident within 1 h of nsPEF treatment, and DNA fragmentation as detected by TUNEL staining was evident by 6 h after nsPEF treatment. In a melanoma allograft system, nsPEF treatment was superior to tumor excision at accelerating secondary tumor rejection in immune-competent mice, suggesting enhanced stimulation of a protective immune response by nsPEF-treated melanomas. This is supported by the presence of CD4⁺-T cells within treated tumors as well as within untreated tumors located in mice with other melanomas that had been treated with nanoelectroablation at least 19 days earlier.

Keywords

Nanoelectroablation; melanoma; apoptosis; pulsed electric field; immune response; allograft tumors; tumor therapy

Introduction

The incidence of cutaneous melanoma is rising in Caucasian populations (Bradford et al., 2010) and it ranks among the top six cancers in both men and women in the United States and Australia. There is good evidence that sunlight exposure increases the risk for developing melanoma as supported by epidemiologic studies of severe episodes of sunburn in early life. Most patients diagnosed with primary melanoma present with stage I disease (Tsao et al., 1998), and the standard of care remains excision of the lesion often accompanied by a sentinel lymph node procedure. We have found that treating melanomas in a subdermal allograft model system with ultrashort, high-voltage electrical pulses can stimulate apoptosis and complete remission over a period of weeks (Nuccitelli et al., 2006, 2009, 2010). To determine whether this nanosecond pulsed electric field (nsPEF) therapy

Conflict of interest

BioElectroMed built the prototype PulseCure Model S-3 used in these experiments but is not marketing this product.

would also be effective on primary UV-induced melanomas in vivo, we have treated a murine model system in which melanomas developed in skin over a period of 5–14 months after the pup's skin was irradiated with UV (Noonan et al., 2001).

This relatively new nsPEF therapy that we are naming 'nanoelectroablation' works by generating transient ionpermeable nanopores in both the plasma membrane (Pakhomov et al., 2007, 2009) and the intracellular membranes (Buescher and Schoenbach, 2003; Tekle et al., 2005) in all of the cells in a treated tumor. The reason that nsPEF can penetrate into cells is that the pulse rise time is faster than the time required for charges to redistribute on the cell's plasma membrane to block penetration of the electric field into the cell (Schoenbach et al., 2004). Molecular modeling indicates that a voltage difference across any organelle membrane of about 1 V is sufficient to drive water molecules into the lipid bilayer to form a narrow, conductive path 1 nm-wide called a nanopore within a couple of nanoseconds (Levine and Vernier, 2010). For a 1 μm -wide organelle, this requirement of 1 V across the membranes at each end means that 2 V/ μm (20 kV/cm) would be required. In practice, we have observed a more consistent response by applying 30 kV/cm. These nanopores allow transmembrane movement of molecules smaller than 300 D (Andre et al., 2010; Pakhomov et al., 2009) as well as a flow of the membrane lipid, phosphatidylserine (PS), from the inner to the outer membrane leaflet (Vernier et al., 2004). The ion flow across the organelle membranes results in an immediate transient mild elevation of intracellular Ca^{2+} , and this is followed by a cascade involving nuclear pyknosis, DNA fragmentation and caspase activation (Schoenbach, 2010; Schoenbach et al., 2006). The PS externalization may be part of the signal that stimulates macrophages to phagocytose the tumor cells (Krysko et al., 2006).

A second advantage of nanoelectroablation therapy is that it is non-thermal in nature because the short pulse duration only introduces 0.4 J/pulse. When a 100-ns-long pulse of 30 kV/cm is applied to a cubic centimeter of tissue with a resistance of 600 ohms, it would raise the tissue temperature by only 0.3°C. This non-thermal mechanism of ablation has a distinct advantage over hyperthermia-inducing radiofrequency ablation because the tissue damage is restricted to the region between the electrodes without any harm to neighboring healthy tissue that would result from the spread of heat (Nuccitelli et al., 2010).

A third advantage of nanoelectroablation therapy is that it triggers apoptosis that has been shown in other systems to generate a potent immune response in vivo (Krysko et al., 2006; Scheffer et al., 2003). Here, we have used an allograft model to generate melanoma tumors by subcutaneously injecting murine B16 melanoma cells to show that secondary tumor growth is inhibited more following nanoelectroablation of the first tumor than it is following surgical excision. In addition, we find CD4^{+} -T cells infiltrating nanoelectroablated tumors within 19 days after treatment.

Results

Melanomas rapidly shrink and disappear after nsPEF treatment

We treated a total of 27 melanomas on 14 different C57/BL6 HGF/SF mice with 2000 pulses 100 ns long and 30 kV/cm at 5–7 pulses per second (Table 1). All treated melanomas remaining in the mice responded by shrinking over a period of 12–29 days until they could no longer be detected. Only three of them required a second nsPEF treatment to be completely eliminated. We used four different electrode configurations (Figure 1), and all appeared to be equally effective in ablating the tumors when a minimum of 20 A was passed through each tumor and the electrode completely enclosed the tumor. The only change in the appearance of the melanoma immediately after nsPEF treatment is some local edema (Figure 1) and epidermal necrosis appearing over the melanoma within a few days (Figure

1D, d8). The melanoma then began shrinking in size and the scab was usually rubbed off by the host within 2 weeks, revealing a pigment-free region of new skin with no signs of the melanoma (Figure 1B, d12). This pigment-free region is quite striking and suggests that nanoelectroablation locally disrupts normal melanogenesis. The new skin became pigmented over the next few months, but the melanoma did not reappear in any of the animals monitored for up to 127 days following nanoelectroablation.

Histology of nsPEF-treated melanomas

Because of the high pigment concentration in the skin of the HGF/SF mice, one cannot image the tumor and its blood supply by transillumination as we have performed with the subdermal allograft model in albino mice (Nuccitelli et al., 2006). Instead, we removed and fixed the treated tumors for histology at various times after nsPEF treatment (Figure 2). We also looked for the presence of amelanotic melanomas by labeling sections with HMB45 before and after nanoelectroablation. We observed staining in all of our untreated melanomas but could detect no staining in treated melanomas fixed 32 days after ablation (Figure 2A,B).

Nanoelectroablation makes the tumor sections appear darker owing to both the coagulation of blood cells in the tumor and an enhanced concentration of pigment. By 12–29 days after treatment, the tumor is no longer visible in histological sections and the new skin is largely pigment-free. We measured the percentage of the histological section occupied by pigment to provide some data in support of this observation (Figure 3A). These results demonstrate that the average pigment density 3 weeks after nsPEF treatment is significantly lower than in normal skin and much lower than in either the treated or untreated tumor. The normal pigmentation of the skin slowly reappeared over the next months and looked normal by around 100 days after treatment (Figure 2J–L).

NsPEF treatment triggers apoptosis

To look for indicators of apoptosis, we removed some nsPEF-treated melanomas at 1, 3, and 6 h after treatment. Condensation of the nuclei, known as pyknosis, was very clear at all of these time points (Figure 3C,D). In addition to pyknosis, we also looked for DNA fragmentation by using TUNEL labeling of bleached histological sections (Figure 4A–D). About 30% of the nuclei showed some DNA fragmentation (brown stain) at 1 and 3 h post-nsPEF treatment but this increased to 73% at 6 h post-treatment, indicating a sharp increase in DNA fragmentation at that time.

Confirming the complete elimination of the melanoma

To document that the treated melanomas were completely eliminated, we conducted a serial section histological analysis of the skin location at which the tumor originally resided prior to nsPEF treatment. We typically sectioned the central 2 mm at 100 μ m-intervals (Figure 4E). We did not detect any residual melanoma cells in any of these serial sections.

Nanoelectroablation stimulates an immune response

To determine whether nanoelectroablation stimulates an immune response, we measured the growth rate of a second injected tumor after treating the first. Melanoma tumors were induced by subcutaneous injection of B16-F10eGFP melanoma cells on the left side of 12 SKH-1 (immunocompetent) and 12 Nu/Nu (immunodeficient) mice. Once the tumors had grown to 4 mm in diameter, the tumors in six SKH-1 and six Nu/Nu mice were nanoelectroablated, while the tumors in the remainder of the mice were surgically excised. A second tumor was induced on the right side of these mice by a second subcutaneous delivery of B16-F10eGFP cells on either 14 or 28 days after the first tumor had been either

nanoelectroablated or surgically excised. Tumor growth was monitored in vivo using both reflected and transilluminated light and by imaging GFP fluorescence.

Tumors grew three times faster in immunodeficient mice than in immunocompetent mice and this difference in growth rate is highly significant ($P = 0.0009$). In immunodeficient mice, the growth rate of the second tumor was similar to that of the first tumor that was either surgically removed or nanoelectroablated (Figure 5). However, in immunocompetent (SKH-1) mice, we observed significant differences between growth rates of the first and second tumor. For melanoma cells injected 14 days after excision of the first tumor, the second tumor grew slightly faster than the first tumor ($P = 0.05$). In contrast to this, second tumor growth was significantly inhibited in mice whose first tumor had been nanoelectroablated ($P = 0.02$). These observations were even more striking in immunocompetent mice in which the second tumor challenge was given 28 days after nanoelectroablation of the first tumor ($P = 0.02$). The second tumor shrank by 70% within 9 days, and GFP was undetectable (Figure 5E). Second tumors in immunocompetent mice in which the first tumor was excised also showed significant growth inhibition compared to the growth of the first tumor ($P = 0.045$) but continued to produce pigment and GFP. The second tumors on day 9 in the 14- and 28-day injected group were significantly different, with the 28-day group having much smaller tumors ($P = 0.003$). Because this growth inhibition was not evident in immunodeficient mice, these results suggest that the immune system is involved in inhibiting the growth of the second tumor and that nanoelectroablation is significantly more effective at stimulating the immune response than tumor excision.

To confirm that an immune response is stimulated by nanoelectroablation, we removed both treated and untreated tumors from the C57/BL6-HGF/SF transgenic mice at various times after treatment and used immunohistochemistry to label CD4⁺-T cells. One melanoma was removed from a mouse that had never been treated with nanosecond pulses and we could not detect any CD4⁺-T cells in the tumor. Similarly, we could not detect CD4⁺-T cells in a melanoma fixed 12 days after nanoelectroablation (Figure 6A,B). However, nucleated CD4⁺-T cells were detected in nanoelectroablated tumors fixed at 19 days post-treatment (Figure 6C,D) and 32 days post-treatment. More importantly, we found them in untreated tumors removed from mice in which another tumor had been treated by nanoelectroablation at either 19 or 32 days before removal (Figure 6E–H). This suggests that the apoptotic, nanoelectroablated tumor stimulated the immune response that elevated CD4⁺-T cells in both treated tumors and untreated ones as long as another tumor in that mouse had been nanoelectroablated.

Discussion

New findings

We have found that UV-induced melanomas can be completely eliminated by treatment with 2000 pulses 100 ns long and 30 kV/cm in amplitude. Apoptosis was indicated by pyknosis of nuclei by 1 h and DNA fragmentation by 3 h after treatment. The amount of DNA fragmentation increased significantly by 6 h after treatment. The treated melanomas were no longer visible 12–29 days after treatment and instead a melanin-free region of skin appeared. Serial sectioning of the skin regions where the treated melanomas had been located revealed a reduction in melanin density with no detectable residual melanoma. This nanoelectroablation therapy may have an advantage over surgical excision in that it appears to enhance stimulation of the immune response, thereby inhibiting second tumor growth in an allograft model. This enhanced immune response was confirmed by the detection of CD4⁺-T cells in both treated and untreated tumors by 19 days after nanoelectroablation of another tumor in the same mouse.

New instrumentation

We limited our study to the treatment of lesions that were 4 mm or less along one axis. This allowed us to use electrodes with an intraelectrode spacing of 4 mm so that a pulse amplitude of 12 kV was sufficient to generate the required electric field of 30 kV/cm. Several previous studies have indicated that this 30 kV/cm is the lower threshold for reliable ablation of skin tumors and initiation of apoptosis (Nuccitelli et al., 2006, 2009, 2010; Beebe et al., 2002; Garon et al., 2007). We built our own pulse generator (PulseCure Model S-3, BioElectroMed Corp., Burlingame, CA, USA) because there were none commercially available with the rise time and amplitude we require to trigger apoptosis in these lesions.

Evidence of apoptosis

The stimulation of apoptosis by nsPEF treatment has been documented in several different cell types both in vitro (Beebe et al., 2002, 2003; Hall et al., 2007; Ren and Beebe, 2011) and in vivo (Chen et al., 2012; Nuccitelli et al., 2006, 2009). Among the first signs of apoptosis are pyknosis and DNA fragmentation. We could detect pyknosis occurring 1 h after nsPEF treatment, just as observed in the subdermal allograft and xenograft tumor models (Chen et al., 2012; Nuccitelli et al., 2006). The TUNEL assay indicated an increase in DNA fragmentation by 3 h after nanoelectroablation with a larger increase by 5 h. Another measure of DNA fragmentation is the alkaline comet assay and this technique has detected DNA fragmentation immediately after nsPEF treatment (Chen et al., 2004; Nuccitelli et al., 2009; Romeo et al., 2011). Several other markers of apoptosis have been observed following nsPEF treatment, including increases in executioner caspases (Chen et al., 2010, 2012; Ford et al., 2010; Ren and Beebe, 2011) and PS externalization (Vernier et al., 2004). This process of programmed cell death takes much longer than necrosis and these UV-induced melanomas began slowly shrinking after nsPEF treatment but were visible for 12–29 days. This provides time for the immune response to detect the apoptotic tumors so we decided to look for an immune response following nanoelectroablation.

Advantages of nsPEF therapy over lesion excision

Because it is well documented that the presence of apoptotic cells elicits a strong CD4⁺- and CD8⁺-T-cell response (Krysko et al., 2006; Scheffer et al., 2003), we used an allograft tumor model to determine whether nanoelectroablation stimulated an immune response. Our results showed that second tumor growth was strongly inhibited and the tumor even shrunk by an average of 70% in immunocompetent mice whose first tumor was nanoelectroablated (Fig. 5F–I). In contrast, lesion excision only slightly inhibited second tumor growth, suggesting a weaker immune system stimulation. The nanoelectroablation-triggered immune response was further supported by immunohistochemistry, showing the presence of CD4⁺-T cells in treated lesions by 19 days following treatment and even in untreated tumors present in animals with another treated tumor. This stimulation of the immune response provides a strong advantage for nanoelectroablation therapy over surgical excision because an activated immune system could inhibit the growth of untreated tumors.

Changes in pigmentation

The most striking change in pigmentation following nanoelectroablation is the initial absence of pigment in the treated region as evident in both the photographs of the skin and the histological sections (Figures 2 and 3). Melanin granules first begin to appear at the periphery of the treated region and slowly fill in over a period of a few months. This could be a concern for the application of this therapy to humans. However, our ongoing clinical trial results from treating human basal cell carcinomas with nsPEF do not indicate such striking changes in pigmentation (Clinicaltrials.gov#NCT01463709).

Conclusion

Nanoelectroablation delivering a field strength of 30 kV/cm can trigger apoptosis and complete remission of UV-induced melanomas in a murine model system (De Fabo et al., 2004; Noonan et al., 2001; Zaidi et al., 2011). These murine melanoma tumors have histological, pathological, and photobiological characteristics remarkably similar to human melanomas. Because the mechanism of nanoelectroablation is a physical one that is not cell-specific, we would expect this therapy to be equally effective in treating human lesions. In support of this, our ongoing clinical trial nanoelectroablating human basal cell carcinomas is yielding excellent results thus far.

Methods

nsPEF pulse generator

Two different 100-ns pulse generators were used in this study. The first (Figure 7) employed a single Blumlein line composed of a 20-m-long coaxial cable in the configuration shown in Figure 7(B). The load and matching resistor were placed at the center of the cable, and the two 10 m-long halves were wound within a cardboard cylinder (Figure 7C). When the combination of load and matching impedance is equal to two times the 50 ohm cable impedance, the full amplitude is delivered with little or no reflection. Because our load impedance is typically 750 ohms, we used a matching resistor of 115 ohms to balance the total impedance to 100 ohms. The second pulse generator used two Blumlein lines in series (also known as a double-Blumlein pulse forming network). These were both 20 m-long, 50 ohm RG-174/U coaxial cables, wound on a cardboard cylinder, and charged to half of the desired delivery pulse amplitude. When the load impedance is equal to four times that of the coaxial cable, the full amplitude is delivered with little to no reflection. As our load impedance was typically 750 ohms, we used a 270-ohm matching resistor in parallel with the load to balance the load to 200 ohms. When discharged through a triggered spark gap at atmospheric pressure, this arrangement delivers a 100-ns-long pulse with a 20 ns rise time (Figure 7D). We used a micro-controller to trigger the spark gap at either 5 or 7 pps, control the voltage level of the power supply, and count the pulses. The pulse counter utilized the signal generated by a custom current sensor placed around one of the wires connected to the suction electrode so that only pulses resulting in current delivery to the tumor were counted.

nsPEF delivery

Each tumor was treated with 2000 pulses at 30 kV/cm using one of four different electrode configurations (Figure 1A–D). Throughout the experiment, the mouse was anesthetized with 1.4% isoflurane through inhalation and was positioned on a warming stage that maintains the mouse's body temperature within the normal range. After applying olive oil or silicone fluid to the tumor surface, an electrode was placed on top of the tumor, and suction was applied within the electrode to pull the tumor into the electrode's 2 mm-deep application region. Tumors were treated with 2000 pulses with a pulse length of 100 ns. If the tumors were treated with a 2-pole parallel plate, the frequency was 7 pps, and if treated with the needle electrode, the frequency was lowered to 5 pps at the same field strength. If tumor regrowth occurred, another treatment was applied until there was no sign of reoccurrence. For tumors longer than 4 mm along one axis (Figure 1D), the electrode was repositioned over the untreated region and an additional 2000 pulses were applied.

Protocol for the induction of melanomas by UV radiation

A detailed protocol for inducing melanomas in mice has been published previously (De Fabo et al., 2004). Briefly, for these experiments, a single exposure of broad spectrum UV radiation from a panel of six FS40 lamps was administered to 3.5- to 4.5-day-old neonates

for a total exposure time of 15 min. At an average irradiance of 10 W/m^2 , a total dose of approximately 9.0 kJ/m^2 is delivered using this UV system (60% UVB/40% UVA/<1% UVC). For comparison with sunlight exposure, see Zaidi et al. (2011).

The UV lamps were mounted 20 cm above the pups, and one pup was placed in each well of a six-well culture dish immediately prior to exposure. As part of the UV rack, a UV blocking curtain (blackout cloth) surrounded the rack and additionally, the back of the UV blocking curtain is left open approximately 1–2 inches to facilitate escape of ozone gas and heat. To prevent ozone buildup, and hence UV absorption, in the UV exposure area, two small (desktop size) fans were mounted diagonally to one another on the shelf and ran continuously throughout the exposure period. Melanomas began to appear about 5–6 months after this exposure (Figure 1E).

Eight male and seven female C57/BL6 HGF/SF transgenic mice, 5–13 months old, were shipped to BioElectroMed and were maintained in In-Vivo Technologies, Inc. (Burlingame, CA, USA) with 12-h light/dark cycle at controlled temperature and humidity (Table 1). Typically, up to four female mice were housed together in the same cage, and each male mouse was housed in individual cages unless they were raised together from birth. Fourteen of these mice were treated and one died before treatment. The mouse numbers in Table 1 were assigned by the De Fabo laboratory and have no relation to the number of mice treated. When each lesion diameter reached 4 mm, the tumor was treated with nsPEF. After receiving treatment, each lesion was photographed every other day. All of the experiments were conducted in accordance with a protocol approved by In-Vivo Technology's Institutional Animal Care and Use Committee.

Histological analysis

The treated tumors were monitored every other day for signs of tumor regrowth and were removed at different time points for histological analysis. Samples of treated tumors and skin were collected after the mice were sacrificed. In cases where the mice possessed multiple tumors, the treated tumors were removed when the mice were under anesthesia, and the wounds resulting from the surgery were sutured. The resected skin samples were immediately fixed in 10% buffered formalin for at least 24 h, were embedded in paraffin, sectioned at $5 \mu\text{m}$ thick, and then stained with H&E. Because of the heavy concentration of melanin in the tumors, melanin bleaching was performed prior to the TUNEL antibody staining to more easily detect the stain. Bleaching involved deparaffinization followed by 0.25% aqueous potassium permanganate for 1 h, followed by 5% aqueous oxalic acid solution for 2–5 min until sections become clear. TumorTACS® In Situ Apoptosis Detection Kit purchased from Trevigen, Inc. (Gaithersburg, MD, USA) was used to recognize cell apoptosis. This kit uses a terminal deoxynucleotidyl transferase-mediated X-dUTP nick end labeling (TUNEL)-based system that labels only the ends of double-stranded DNA fragments found in apoptotic cells. DNA fragments generated during apoptosis are end-labeled with modified nucleotides using a highly purified terminal deoxynucleotidyl transferase enzyme (TdT) in a non-toxic labeling buffer. The incorporated nucleotides are subsequently detected using a horseradish peroxidase conjugate. The conjugate catalyzes the conversion of diaminobenzidine (DAB) into a visible dark brown precipitate to indicate that the cells are apoptotic.

Immunohistochemistry

Immunohistochemistry was carried out on paraffin-embedded sections of melanomas fixed in buffered 10% formalin. Deparaffinization in the standard xylene and ethanol series was carried out prior to antibody labeling. The anti-melanoma antibody, HMB 45, was obtained from Abcam (Cambridge, MA, USA, ab732), and 3-amino-9-ethylcarbazole was used as a

chromogen, generating a rust color when combined with melanin. Antibodies to CD4 and CD8 were obtained from R&D Systems (Minneapolis, MN, USA, MAB116, MAB554). The CD8 antibody did not label our paraffin-embedded sections (including the positive control spleen sections), but the CD4 antibody did. Therefore, we used only the anti-CD4 antibody to identify CD4⁺-T cells combined with a goat anti-rat secondary conjugated to Alexa Fluor 488. DAPI dye was added to stain nuclei. We followed the protocol for fluorescent staining of paraffin-embedded sections provided by R&D Systems.

Measuring pigment density

The 200- μm -thick region beneath the epidermis was evaluated using Image J (downloaded from www.nih.gov). A fixed surface area ($1000 \times 200 \mu\text{m}$) on each of the H&E-stained section was analyzed, and the total area, the integrated density, and the percentage of the area consisting of the dark brown pigment were determined for normal skin, untreated melanoma, and treated melanoma at two time points after nsPEF treatment.

Serial sections of nsPEF-treated region after tumor regression

To confirm that the nsPEF-treated tumors were completely eliminated, we fixed and serial sectioned the skin region in which the tumor was originally located. A 5- μm -thick section was taken every 100 μm throughout the entire region for several of the treated tumors ranging from 14 to 101 days following treatment (Table 1).

Statistical analysis

Data are presented as mean $\pm$ SEM, and the level of significance is determined by a paired t test.

Methods for measuring growth inhibition of a second tumor in allograft model

Cell line—B16-GFP melanoma cells transfected with green fluorescent protein (eGFP) were obtained from Dr. Alan Houghton at the Memorial Sloan-Kettering Cancer Center on 23 November 2008 and stored in liquid nitrogen until use. Frozen vials were thawed and cultured in alpha minimum essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin–streptomycin. Cultures were maintained in a 5% CO_2 incubator at 37°C.

Animals used in allograft experiments—Twelve female SKH-1 immunocompetent mice (hairless albino) and 12 female Nu/Nu immunodeficient mice (hairless albino) from 4 to 6 weeks old purchased from Charles River (Hollister, CA, USA and Wilmington, MA, USA) were used in this study. All animals were housed at 22°C with a 12-h light/dark cycle. All animal care for these allograft experiments was performed by the BioElectroMed animal facility, and protocols were approved by BioElectroMed's IACUC. Mice were anesthetized by inhalation anesthesia using 1.4% isoflurane in O_2 and positioned on a 37°C warming bed during each procedure.

Tumors—Twelve Nu/Nu and 12 SKH-1 mice were injected with 10^6 B16-GFP melanoma cells in 15 μl of Hank's buffered salt solution (HBSS) using a Hamilton syringe while under inhalation anesthesia. A total of two such injections were administered into each mouse. Tumors were visually detected by the small bulge formed and by transillumination photography. Photographs were taken with a bright field, transillumination, and fluorescent microscopy when visible. Fluorescent microscopy was taken with a manual exposure time of 0.25 s. Photographs were taken at least every other day. A parallel plate suction electrode, with a 4-mm gap, was used for nsPEF treatment throughout the allograft experiments.

Tumor removal—For the control groups, the first tumor was surgically excised prior to the second tumor cell injection. All mice were anesthetized with 1.4% isoflurane inhalation anesthesia and positioned on a 37°C warming bed prior to the removal of a tumor. The surface was cleaned with an antiseptic germicide, povidone–iodine swab stick followed by an alcohol pad. All surgical instruments were autoclaved prior to the surgery. The tumor was then lifted with a 4-SA technic tweezers, and a slit was then made around the tumor with a 25-mm straight edge Fine Science Tools scissors. After removing the tumor, the wound was closed using a Vantage V7-2 forceps with an Ethicon P-3, 5-0 Ethilon black nylon monofilament surgical suture. The sutures were removed after 1 week if the sutures were still intact.

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Figure 1.

Typical melanoma responses to 2000 pulses, 30 kV/cm, 100 nanosecond pulsed electric field (nsPEF) treatment using four different suction electrode configurations. ‘–’ indicates tumor appearance before nsPEF treatment and ‘+’ indicates tumor appearance just after treatment. Images below that show the tumor appearance on the indicated day after treatment. (A) Parallel plate electrode as used on this tumor. (B) Hemicircular electrode treatment; (C) Six-pole dual electrode treatment; (D) Six-needle electrode in parallel array. (E) Typical melanomas on a C57/BL6 HGF/SF mouse.

Figure 2.

Histological changes in treated melanomas. (A) Unbleached section of untreated tumor labeled with the rust-colored melanoma marker, HMB45 (arrows). (B) Unbleached section of nanoelectroablated melanoma 32 days after treatment shows no labeling with HMB45. (C) Two sections through normal skin labeled with H&E. (D) Two sections through an untreated melanoma labeled with H&E. (E) Matched pair of a histological section through the center of the lesion and a surface view at 1 h after nanoelectroablation. (F) Image pair taken 6 h after nanoelectroablation. (G) 24 h after nanoelectroablation. (H) Seven days after nanoelectroablation. (I) Twelve days after nanoelectroablation; (J) 28 days after nanoelectroablation. (K) Seventy-five days after nanoelectroablation. (L) One hundred and twenty-seven days after nanoelectroablation. There is no evidence of a tumor by day 28, although the skin pigmentation is not normal. By 100 days after treatment, the pigmentation looks similar to the untreated control skin.

Figure 3.

Changes in pigmentation following nanosecond pulsed electric field (nsPEF) treatment. (A) Comparison of pigment areas within the rectangular boxes on histological sections taken at different times following nanosecond pulsed electric field (nsPEF) treatment. The number of samples within each mean area is provided, and the bars indicate SEM. (B) H&E-stained section from an untreated melanoma. (C, D) Pyknosis observed 1 h after nsPEF treatment. H&E-stained sections from a nsPEF-treated melanoma fixed 1 h after treatment with 2000 pulses of 30 kV/cm. To facilitate visualization of pyknosis, several nuclei were outlined and copied in Photoshop and placed just beneath their sections. These three images were taken at the same magnification indicated by the scale bar in 'B'.

Figure 4.

(A–C) TUNEL labeling of bleached histological sections of nanosecond pulsed electric field (nsPEF)- treated melanomas. (A) Section prepared 1 h after nsPEF treatment. (B) Section prepared 3 h after nsPEF treatment. Arrows point to brown nuclei. (C) Section prepared 6 h after nsPEF treatment. Many brown nuclei are present. Scale bar in ‘A’ applies to ‘B’ and ‘C’ as well. (D) Percentage of nuclei staining blue (no fragmentation) or brown (DNA fragmentation). (E) Unbleached serial sections at 100-μm intervals through a skin region where a melanoma was located. Skin was removed 101 days after nsPEF treatment of a 27 mm² melanoma on mouse No. 7. Number on each image indicates that section’s position in microns within the tissue block. The scale bar in ‘E’ applies to all of these serial sections.

Figure 5.

Secondary tumor growth is inhibited following nanoelectroablation of the primary tumor. Each group of three images is composed of a reflected light surface view on the left, a transilluminated view of the tumor in the center, and a fluorescent image on the right. The day after injection on which the photographs were taken is indicated in upper left of the reflected light image. (A) The typical growth of the first melanoma injected into either Nu/Nu or SKH-1 mice. (B) Second tumor growing from cells injected 14 days after the first tumor was removed by surgical excision in both genotypes. (C) Second tumor growing from cells injected 28 days after the first tumor was removed by surgical excision in both genotypes. (D) Second tumor growing from cells injected 14 days after the first tumor was treated with nanoelectroablation in the two genotypes. (E) Second tumor growing from cells injected 28 days after the first tumor was treated using nanoelectroablation. This experiment

was repeated three times with similar results, and the mean tumor areas are plotted below. (F) Immunosuppressed mice in which the second tumor was injected 14 days after removal or nanoelectroablation of the first. (G) Immunosuppressed mice in which the second tumor was injected 28 days after removal or nanoelectroablation of the first. (H) Immunocompetent mice in which the second tumor was injected 14 days after removal or nanoelectroporation of the first. (I) Immunocompetent mice in which the second tumor was injected 28 days after removal or nanoelectroporation of the first. Each mean is an average of three separate experiments, and the bars represent SEM.

Figure 6.

CD4⁺-T cells in melanoma tumors. (A) Unstained, unbleached thin section of a melanoma fixed 12 days after nanoelectroablation treatment. Scale bar represents 200 μ m and applies to all parts of this figure. (B) Fluorescent label from CD4 antibody applied to 'A' indicates surface labeling but no T cells could be found within the tumor. (C) Unstained, unbleached thin section from a melanoma fixed 19 days after nanoelectroablation. (D) Superposition of both Hoechst (blue) and anti-CD4 (green) fluorescence from the section shown in 'C' indicates the presence of CD4⁺-T cells (arrows) within the tumor. (E) Unstained, unbleached thin section of an untreated melanoma from a mouse in which another melanoma had been nanoelectroablated 19 days earlier. (F) Superposition of Hoechst (blue) and anti-CD4 (green) fluorescent label from section shown in 'E' indicates the presence of CD4⁺-T cells (arrows) within the untreated tumor. (G) Unstained, unbleached section from an untreated melanoma from a mouse in which another melanoma was nanoelectroablated 32 days earlier. (H) Superposition of Hoechst (blue) and anti-CD4 (green) fluorescent label from section shown in 'G' indicates the presence of CD4⁺-T cells (arrows) are found within this untreated tumor.

Figure 7.

PulseCure Model S-1 is the 100-ns pulse generator used in this study. (A) Photograph of pulse generator and mouse warming stage with electrode holder above it. (B) Circuit diagram of the 100 nanosecond pulse generator used here. (C) 3D model showing the arrangement of the components inside the box in 'A'. The two 10-m-long RG-213 coaxial cables were wound on the inner wall of the cylindrical tubes. (D) Typical oscilloscope trace of the waveform generated by the pulse generator. Twelve kilovolts was applied to suction electrodes with 4 mm between the plates so that the tumors experienced 30 kV/cm and current varied between 12 and 40 A depending on the electrode type and skin resistance.

Table 1

List of mice and treated melanomas

Mouse No.	Lesion No.	Tumor size (mm)	W × L, Area (mm ²)	Age (months)	Electrode type	pps [/]	Day no longer visible ²	Day tumor removed ²	% Area pigmented
10	2	6.37 × 3, 20.44		16	Needle	5	–	p1d0 1 h	77.226
10	5	4.14 × 2.88, 12.35		16	Needle	5	–	p1d0 3 h	44.214
86	2	3.99 × 2.85, 12.64		11	Needle	4	–	p1d0 3 h	67.664
36	1	4.78 × 4.43, 25.26		12	Needle	5	–	p1d0 6 h	77.648
94	2	4.33 × 3.27, 12.99		12	Needle	5	–	p1d0 6 h	79.543
36	4	3.15 × 3.42, 9.78		14	Parallel	5	–	p1d1 24 h	73.728
36	7	3.88 × 2.99, 7.86		14	Parallel	5	–	p1d1 24 h	52.413
94	6	4.38 × 3.26, 12.96		13	Parallel	7	–	p1d7	11.125
78	3	4.09 × 2.74, 12.04		14	Parallel	5	–	p1d12	0.009
86	5	10.12 × 3.23, 31.34		13	Parallel	5	p1d12	p1d12	0.54
11	5	6.29 × 3.81, 24.72		10	6-pole	7	p1d19	p1d19	0.177
78	4	3.87 × 2.38, 10.29		13	Parallel	5	p1d19	p1d19	0.0005
86	4	4.41 × 3.51, 14.24		12	Parallel	5	p1d20	p1d20	0.017
78	2	4.49 × 3.21, 14.85		13	Parallel	5	p1d22	p1d28	3.634
86	3	4.11 × 3.19, 12.23		12	Parallel	7	p1d22	p1d28	0.886
40	1	3.28 × 2.83, 8.43		7	Hemi	7	p1d29	p1d29	0.048
48	1	3.59 × 3.6, 12.37		9	Hemi	7	p2d18	p1d31	0.271
87	3	6.41 × 4.62, 25.31		9	Parallel	7	p1d19	p1d32	3.975
7	2	3.16 × 3.54, 11.25		13	Hemi	7	–	p2d45	0.068
35	1	4.74 × 3.35, 16.03		6	Hemi	7	p2d48	p2d48	0
324	1	4 × 3.07, 10.62		7	6-pole	7	p1d14	p1d50	7.834
84	1	10.34 × 5.55, 51.94		8	Needle	7	p1d19	p1d75	1.0575
7	1	6.86 × 4.58, 26.92		13	6-pole	7	p2d76	p2d101	1.613
86	1	4.23 × 3.04, 14.49		9	Parallel	7	p1d22	p1d127	18.76
7	3	3.71 × 2.69, 10.15		13	6-pole	7	–	–	–
7	6	3.24 × 3.92, 11.71		13	Hemi	7	–	–	–
48	5	2.53 × 0.94, 3.57		10	6-pole	7	–	–	–

[/] pps, pulses per second;

² the numbers after p refer to the number of nsPEF treatments and the numbers following d refer to the number of days after the first nsPEF treatment.