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In vitro and *in vivo* evaluation and a case report of intense nanosecond pulsed electric field as a local therapy for human malignancies

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When delivered to cells, very short duration, high electric field pulses (nanoelectropulses) induce primarily intracellular events. We present evidence that this emerging modality may have a role as a local cancer therapy. Five hematologic and 16 solid tumor cell lines were pulsed *in vitro*. Hematologic cells proved particularly sensitive to nanoelectropulses, with more than a 60% decrease in viable cells measured by MTT assay 96 hr after pulsing in 4 of 5 cell lines. In solid tumor cell lines, 10 out of 16 cell lines had more than a 10% decrease in viable cells. AsPC-1, a pancreatic cancer cell line, demonstrated the greatest *in vitro* sensitivity among solid tumor cell lines, with a 64% decrease in viable cells. When nanoelectropulse therapy was applied to AsPC-1 tumors in athymic nude mice, responses were seen in 4 of 6 tumors, including clinical complete responses in 3 of 6 animals. A single human subject applied nanoelectropulse therapy to his own basal cell carcinoma and had a complete pathologic response. In summary, we demonstrate that electric pulses 20 ns or less kill a wide variety of human cancer cells *in vitro*, induce tumor regression *in vivo*, and show efficacy in a single human patient. Therefore, nanoelectropulse therapy deserves further study as a potentially effective cancer therapy.

Key words: nanoelectropulse therapy; pancreatic cancer; basal cell carcinoma

Medical care of cancer patients requires therapeutic modalities that are both local and systemic. A striking example of a local cancer therapy is surgical resection, the most successful tool in oncologic therapy. Many other local therapies, including radiation therapy,[1] radiofrequency ablation,[2] high-intensity focused ultrasound,[3] microwave thermal therapy,[4] laser thermal ablation,[5] cryotherapy[6] and tumor embolization,[7] fulfill roles from palliation to curative treatment. Each of these therapies is limited by suboptimal efficacy and damage to normal tissues. Approaches in which electrical energy is delivered to a tumor have also been attempted, generally as part of an attempt to induce electroporation.[8] For instance, after intralesional injection of bleomycin, a single course of 1.3 kV/cm pulses achieved complete response in 94% of human basal cell carcinomas.[9]

Another modality in development is nanoelectropulse therapy. This therapy utilizes ultra-short (nanosecond), high-field (10 kV/cm range) electric pulses designed to bypass the cell membrane capacitance, reaching the cell interior with intense electric fields.[10] Properly delivered nanosecond electrical pulses, with high instantaneous power but very low total energy, initiate events that are distinctly intracellular.[11] Effects include externalization of phosphatidylserine,[12–14] release of intracellular calcium,[15,16] mitochondrial release of cytochrome c[17] and caspase activation.[11,18] Surveys of the sensitivity of various malignant cells have been initiated,[19–21] but only a few cell types have been tested at this time. Recently, it has been shown that complete remission in melanoma xenografts can be achieved after 2 courses of nanoelectropulses, with a resultant temperature increase of only 3°C.[22] Previously reported *in vivo* observations with human tumor cells have involved relatively long pulses,[8,14,22] compared to the 20 ns pulses used in our experiment. Pulses of shorter duration theoretically deliver perturbative energy more efficiently to the contents of the cell without destructively disrupting the plasma membrane.

Some of the work in this study focuses on pancreatic cancer and basal cell carcinoma. Pancreatic cancer is a particularly aggressive malignancy with less than 5% of patients alive 5 years after diagnosis.[23] Other than surgery, which is rarely possible, radiation is the only local therapy commonly used in this malignancy. However, the largest study of patients treated with radiation therapy in the adjuvant setting demonstrated increased mortality when radiation was used.[24] In contrast to pancreatic cancer, basal cell carcinoma is remarkably indolent. It represents 80% of non-melanomatous skin cancers, making it the most common human malignancy. Fortunately, metastases occur in less than 1% of all patients.[25] So, essentially all therapy is directed at the local lesion. Although surgery is associated with low rates of recurrence, many patients prefer to avoid surgery, and some patients are poor surgical candidates. Therefore, improved non-surgical local therapies would be very helpful in both malignancies.

In this study, we evaluated the ability of nanoelectropulse therapy to kill or impede the growth of malignant cells. We sought to determine optimal *in vitro* pulsing regimens using the Jurkat T cell leukemia/lymphoma cell line, a cell line known to be very sensitive to nanoelectropulsing.[26] We tested human cancer cell lines from multiple types of malignancies *in vitro* to determine the responsiveness of cancer cell lines from different malignancies. We evaluated responses between normal and malignant cells by using WI-38, a cell line established from normal human fetal lung fibroblasts, and VA-13, the matched WI-38 cells transformed with the SV40 virus (which inactivates p53 and Rb). We also evaluated *in vivo* nanoelectropulses by determining optimal pulsing regimens and then assessing the efficacy of this approach in a sensitive cell line. Finally, one of the investigators applied nanoelectropulse

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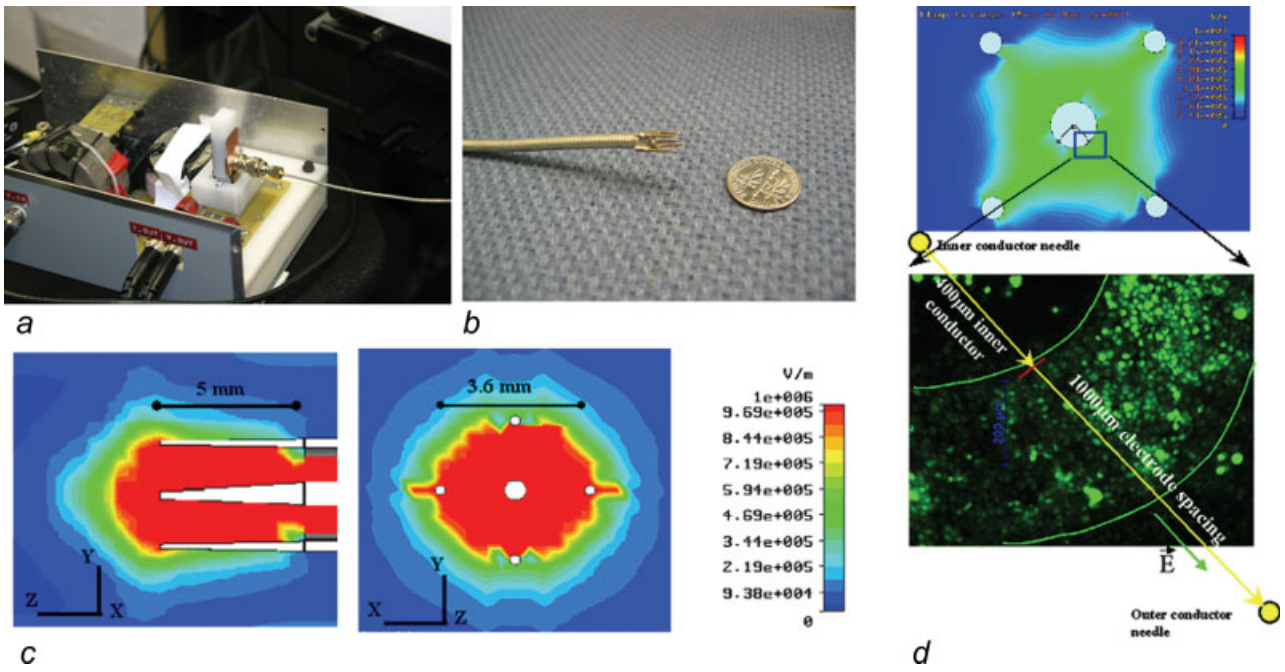

FIGURE 1 – Pulse generator and catheters for *in vivo* experiments. Pulse generator (*a*) and experimental catheter (*b*) used in the *in vivo* experiments. (*c*) Simulated electric field distribution in RPMI medium pulsed using the catheter. The color ramp is set up to 10 kV/cm, assumed threshold electric field for intracellular response of cells. (*d*) Simulated electric field distribution (above) and fluorescence images (below) of SKOV-3 ovarian cancer cells pulsed with 150 pulse of 20 ns duration at 60 kV/cm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

therapy to his own basal cell carcinoma to assess whether a response could occur in a human malignancy.

Material and methods

Cell lines and cell cultures

The Jurkat and RPMI 8226 cell lines were purchased from ATCC (Manassas, VA). SUDHL-6, SUDHL-16 and OCI-Ly4 were generous gifts from Sven DeVos at UCLA. These cell lines were grown in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 50 IU/ml penicillin and 50 mg/ml streptomycin (RPMI). PANC-1, HPAC, MIA PaCa-2, AsPC-1, BxPC-3, H520, H460, MCF-7, LNCaP, MDA-MB-231, HCT-116, U-87 MG, SKOV-3, WI-38 and WI-38 VA-13 subline 2RA (VA-13) were purchased from ATCC (Manassas, VA). The U-343 MG line was a generous gift from Keith Black at Cedars-Sinai Medical Center. These cell lines were grown in Dulbecco's modified essential medium containing 10% heat-inactivated fetal calf serum (FCS), 50 IU/ml penicillin and 50 mg/ml streptomycin (DMEM). They were separated from plates using trypsin-EDTA.

Pulse generation for in vitro *studies*

A MOSFET-based, inductive-adder pulse generator with a balanced, coaxial-cable pulse forming network and spark-gap switch designed and assembled in the Pulsed Power Science laboratories at the University of Southern California delivered electrical pulses with approximate rise and fall times of 2–3 ns to cell suspensions in electroporation cuvettes. Total duration of pulse was determined by the length of coaxial cable, resulting in pulses of 7, 10, and 20 ns duration with plateaus of 2, 5, and 15 ns respectively.

Experimental evaluation of electric field

SKOV-3 cells were cultured in a coverglass chamber and loaded with 5 μM YOPRO-1. The cells were pulsed with 150 pulses of 20 ns duration, and 60 kV/cm. Cells were then observed under the Zeiss Axiovert 200 M fluorescence microscope.

In vitro *application of nanoelectropulses to cells in cuvettes*

All cell lines were exposed to nanoelectropulses under identical conditions. A total of 1×10^6 cells were placed in 85 μl of the appropriate growth medium and suspended in a rectangular electroporation cuvette with 1 mm electrode separation. Cells received pulses of 7, 10 or 20 ns duration delivered at a frequency of 20 Hz, and an average field of 25 or 35 kV/cm. Average field was determined by applied voltage divided by electrode separation (1 mm). After nanoelectropulsing, cells were removed from the cuvette and resuspended in 1 ml of the appropriate growth medium. About 10 μl were removed and mixed with 30 μl of trypan blue (Sigma-Aldrich, St. Louis, MO). Of the remaining cells, 2×10^3–2×10^4 cells (standardized for each cell line) were placed in 100 μl of culture medium. Each cell line was plated in a total of 9 wells of a 96 well plate, with 3 wells on 3 separate plates. Experiments were performed twice in most cell lines described, and 3 times for Panc-1 and AsPC-1.

Assessment of cell viability

Cell viability was assessed 96 hr after nanoelectropulsing. At each time point, 10 μl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide][27] was added to each well of a 96-well plate. Plates were incubated at 37°C for 4 additional hours. Then, 50 μl of 20% SDS was added to the wells to permeablize cell membranes. Plates were incubated for 20 additional hours at 37°C. Plates were read at an emission wavelength of 540 nm on an Elisa plate reader, with higher optical density indicating greater uptake of the MTT and a higher number of viable cells.

Pulse generation for in vivo studies

Catheter electrodes for tumor treatment were driven by a MagnePulser I magnetic compression pulse generator with a diode

opening switch designed and assembled in the Pulsed Power Science laboratories at the University of Southern California (Fig. 1*a*). The electrode assembly consisted of a center conductor pin and an array of 4 gold-plated brass pins (0.02 in × 0.125 in) aligned axially and soldered equidistant around the end of the outer conductor of a 50 Ω coaxial cable (PE-402SL, Pasternack Enterprises) (Fig. 1*b*).

In vivo *application of nanoelectropulses to tumors in mice*

AsPC-1 cells were suspended in matrigel at a concentration of 1×10^7/ml. A total of 1×10^6 cells were injected subcutaneously in the flank of a female athymic nude mouse. Once tumors were visible (generally about 10 days), animals were anesthetized with isoflurane, a 7 mm skin incision was placed over the tumor, and a catheter was inserted through the incision, with the prongs resting in the tissue surrounding the tumor. A total of 6.5 kV pulses were delivered at a rate of 20 Hz. Number of pulses delivered ranged from 65 to 1,000. In the final cohort of treated mice, 6 animals received 3 bursts of 75 pulses delivered with 60 sec between each burst of pulses. Pulses were delivered on 3 non-consecutive days over a 5-day period. Tumors were measured with calipers in 2 dimensions, 3 times each week. In 3 animals, incisions were placed and the catheter was inserted into tissue surrounding the tumor, but no pulses were delivered (mock pulsed). After animals were sacrificed, the tumors were dissected and weighed. Tumors were placed in 10% neutral buffered formalin for 24–48 hr and then transferred to 70% ethanol. The tumors were embedded in paraffin, sectioned and evaluated morphologically by light microscopy after staining with Hematoxylin and Eosin.

Data analysis for in vitro *and* in vivo *mice experiments*

In vitro. The average optical density at 540 nm (OD 540) of empty wells was subtracted from the value of each well containing cells. Using these adjusted values, the OD 540 of wells containing pulsed cells was divided by the average OD 540 for unpulsed cells of the same cell line, yielding a percentage of control. Error bars correspond to standard error.

In vivo. Average area for tumors receiving identical therapies was calculated. Error bars correspond to the standard error. Average weight for tumors receiving identical therapies was calculated. Error bars correspond to the standard error.

Temperature calculations for in vitro *and* in vivo *experiments*

All assumptions were chosen to bias calculations towards greater heat generation than could actually occur under non-ideal circumstances.

In vitro. We assumed that all of the pulse voltage applied to the cuvette electrodes results in ohmic current through the cell suspension without significant voltage drops at the electrode-medium interfaces or transfer of heat to the environment.

$E = P \times t = E = (V^2/R)nt$ where V is voltage delivered, R is impedance, n is number of pulses, and t is time. The estimated cell suspension impedance is 10 Ωs. So, for 200 pulses with a peak voltage of 3.5 kV, $E = (3500^2/10) \times 200 \times (2 \times 10^{-8}) = 4.9$ J = 1.2 cal. Approximating the specific heat and mass of the 85 μl cell suspension as 1 cal/g°C and 85 mg, if all of the pulse energy were converted to heat, the temperature would increase 14°C (1.2/0.085).

In vivo experiments, the SAR (specific absorption rate) is expressed as: $SAR = \sigma \frac{E^2}{\rho} = C \frac{\Delta T}{\Delta t}$ where σ is the electric conductivity of tumor tissue in S/m, E is the electric field in V/m, ρ is the tumor's density in kg/m^3, C is the specific heat of tumor tissue in W sec/(kg°C), ΔT is the temperature rising, Δt is the brief time duration of power delivery. The estimated values are: $\sigma = 0.5$, $\rho =$ 1,060 and C = 3,600. The distance between the inner and outer needles is about 1.5 mm for the catheter used in the experiment. The peak voltage delivered is about 6.5 kV. So, with the assumption that all energy is transferred to the pulsed area, no heat transfers between pulsed tumor and surrounding tissue, for 75 pulses, $\Delta t = 20$ ns × 75 = 1.5 μs and $\Delta T = 3.7$°C.

Case study of nanoelectropulse application to a human basal cell carcinoma

A dermatologist diagnosed an 8 × 4 mm^2 basal cell carcinoma on one of the investigators during a physical examination. The investigator then developed a study/treatment plan to apply nanoelectropulses to his own basal cell carcinoma. First a biopsy was obtained to confirm the diagnosis. After applying EMLA as a topical anesthetic, the investigator delivered 200 pulses (10 pulse bursts every 5 sec) to the remaining basal cell carcinoma using the catheter previously described. Pulses were 6.5 kV, 20 ns duration, and delivered at 20 Hz. Six weeks after pulse delivery, the skin that had contained the basal cell carcinoma was surgically excised and pathologically evaluated. Retroactive institutional review board exemptions were obtained at both Cedars-Sinai Medical Center and the University of Southern California.

Results

Selection of nanoelectropulse regimen

We used the MTT assay to assess viable Jurkat human T-cell leukemia/lymphoma cells 96 hr after nanoelectropulse therapy. A total of 200 pulses of 20 ns duration at 25 kV/cm led to an 84% decrease in viable cells compared to controls. In a regimen with equivalent energy, 570 pulses of 7 ns duration at 25 kV/cm produced only a 20% decrease in viable cells. A total of 200 pulses of 20 ns duration at 35 kV/cm caused complete eradication of the cells (Fig. 2*a*). Trypan blue assays demonstrated little cell death from immediate destruction of the cell membrane (less than 2% of cells were trypan blue positive) in all the cell lines under all the pulsing conditions evaluated (data not shown).

In vitro *viability after nanoelectropulses*

Non-Jurkat, human hematologic cells. At 96 hr after pulsing with 200 pulses of 20 ns duration at 35 kV/cm, the multiple myeloma cell line RPMI 8226 had a 73% decrease in viable cells compared to controls. SUDHL-6, a follicular lymphoma cell line, and SUDHL-16, a diffuse large B cell lymphoma cell line, had 61% and 65% decrease in viable cells, respectively. In contrast, OCI-Ly4, another diffuse large B cell lymphoma cell line, had only a 10% decrease in viable cells (Fig. 2*b*).

Pancreatic cancer cell lines. After pulsing 10 solid tumor cell lines, Panc-1, a pancreatic cancer cell line, experienced the greatest response with a 40% decrease in viable cells compared to controls at 96 hr after 200 pulses of 20 ns duration at 35 kV/cm. Therefore, four additional pancreatic cancer cell lines were pulsed under identical conditions, with only HPAC being refractory to this pulsing regimen. BxPC-3, MIA PaCa-2 and AsPC-1 had an 11%, 41% and 64% decrease in growth, respectively, at 96 hr after pulsing (Fig. 2*c*).

Moderate and non-responsive solid tumor cell lines. The lung cancer cell line H520, the ovarian cancer cell line SKOV-3 and the brain cancer cell line U343 had a 14–18% decrease in viable cells compared with controls, 96 hr after 200 pulses of 20 ns duration at 35 kV/cm. The breast cancer cell line MCF-7 had a 30% decrease (Fig. 2*d*). Several solid tumor cell lines had no significant response to 200 pulses of 20 ns duration at 35 kV/cm, including the prostate cancer line LNCaP, breast cancer line MDA-MB-231, colon cancer line HCT 116 and brain cancer line U87. Some refractory cell lines were sensitive to more aggressive pulsing regimens. For instance, HCT 116 had more than a 90% decrease in viable cells after 1,000 pulses of 20 ns duration at 35 kV/cm (data not shown).

Response to pulsing in wild type and transformed WI-38 cells. The matched cell lines WI-38 and VA-13 received 200,

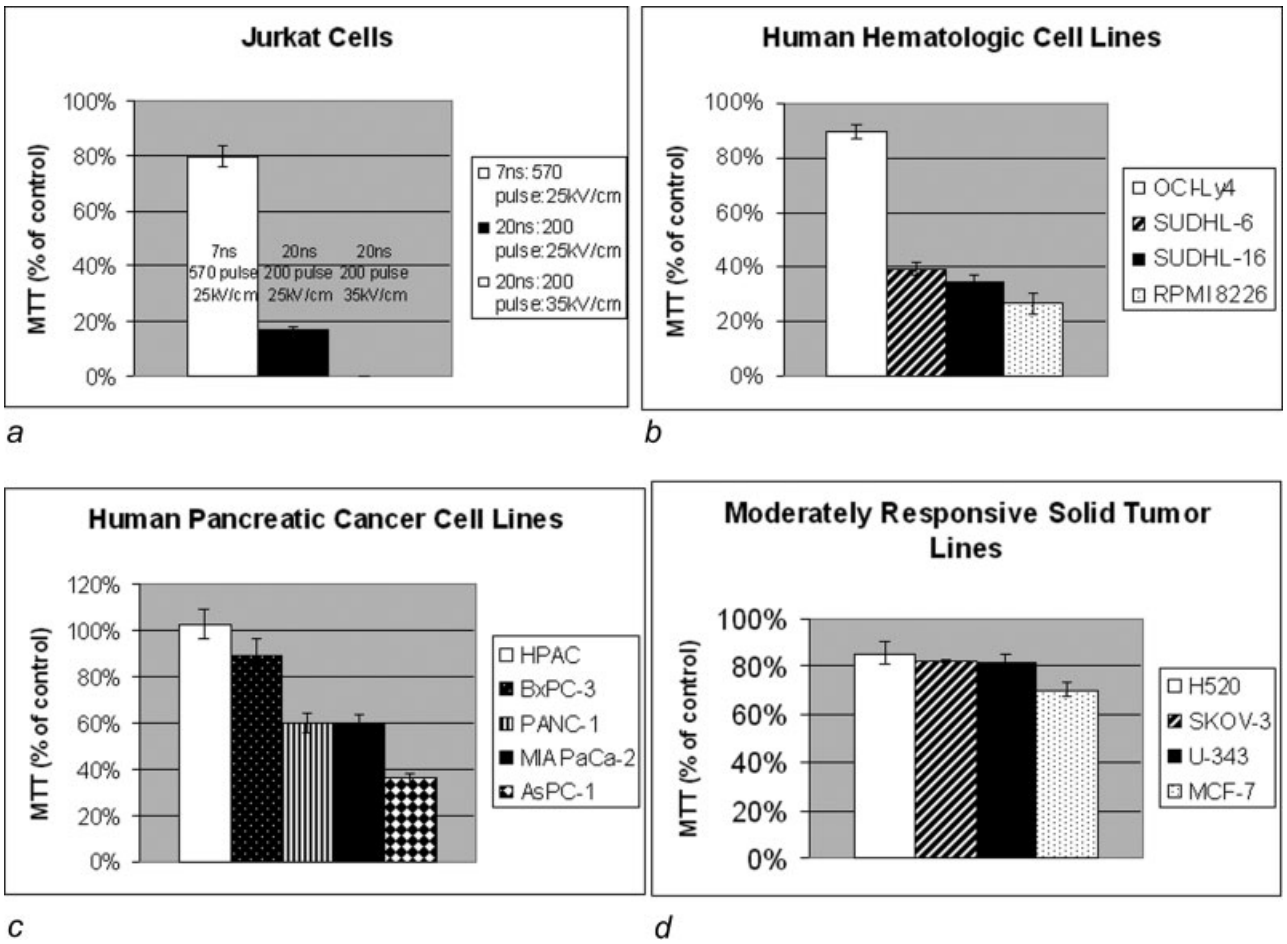

FIGURE 2 – Human cancer cell lines exposed to nanoelectropulsing. Jurkat human T cell leukemia/lymphoma cells received either 570 pulses of 7 ns duration at 25 kV/cm, 200 pulses of 20 ns duration at 25 kV/cm or 200 pulses of 20 ns duration at 35 kV/cm (*a*). 200 pulses of 20 ns duration at 25 kV/cm were delivered to the diffuse large B cell lymphoma cell lines, OCI-Ly4 and SUDHL-16; the follicular lymphoma cell line, SUDHL-6; and multiple myeloma cell line, RPMI 8226 (*b*), the pancreatic cancer cell lines HPAC, BxPC-3, Panc-1, MIA PaCa-2 and AsPC-1 (*c*), the lung cancer cell line H520, ovarian cancer cell line SKOV-3, brain cancer cell line U-343 and breast cancer cell line MCF-7 (*d*). Cells were cultured for 96 hr, their numbers were measured by MTT, and results were compared to sham-treated control cells. Results represent the mean and standard error of 9 wells.

300 or 500 pulses of 20 ns duration at 35 kV/cm. After 200 pulses, the decrease in proliferation was greater in the WI-38 normal fibroblast line (43% *vs*. 31%); whereas after 300 or 500 pulses, the decrease was greater in the VA-13 transformed fibroblast line, 70% *vs*. 55% after 300 pulses, and 86% *vs*. 64% after 500 pulses (Fig. 3).

Electric Field Distribution for in vivo *experiments*

The electric field at the catheter tip has been studied with both simulations (principally Microwave Studio) and experiments in order to determine the distribution and variation in field strength. By simulated analysis, field strength was maximal at the center (high voltage) electrode. Nonetheless, the intense field extends to the grounded exterior pins, and the volume within the catheter pin periphery was essentially filled with the field (Fig. 1*c*). The evenly distributed outer needles confined the high electric field inside the volume surrounded by them. Once signal propagated beyond those electrodes, it decayed quickly. This simulation result was supported by experimental studies, where the electric field distribution was investigated in pulsed SKOV-3 ovarian cancer cells. The cells affected by the nanosecond electric field pulses correlated well with the simulation (Fig. 1*d*).

In vivo *response of pancreatic cancer tumors in mice to nanoelectropulses*

To optimize pulse delivery, various catheters and pulsing regimens were examined in a total of 13 mice. The most effective conditions for delivering pulses with good efficacy and without arcing of the electricity included creation of a small skin incision, insertion of a catheter with prongs in the tissue surrounding the tumor, and delivery of a total of ~200 pulses of 6.5 kV at a 20 ns duration (data not shown). During this portion of the study, tumors were histologically analyzed from mice that were euthanized 24 hr after pulsing. This histologic evaluation revealed 20–60% hemorrhagic necrosis in the pulsed tumors (Fig. 4). Maximal necrosis was seen in tumors in which 200 pulses were delivered

When 9 additional mice were evaluated (6 pulsed, 3 mock pulsed), differences were observed between the 6 pulsed and 3 mock pulsed tumors (Fig. 5*a*). Pulses were effectively delivered, with arcing in only 14% of pulsing sessions. Tumor shrinkage was seen in 4 of the 6 pulsed tumors. Three of 6 pulsed tumors experienced complete clinical response, defined as absence of any visible or palpable tumor for a period of at least 4 days after application of the pulses. A representative growth curve from one of the responding animals is shown, contrasting the pulsed tumor and

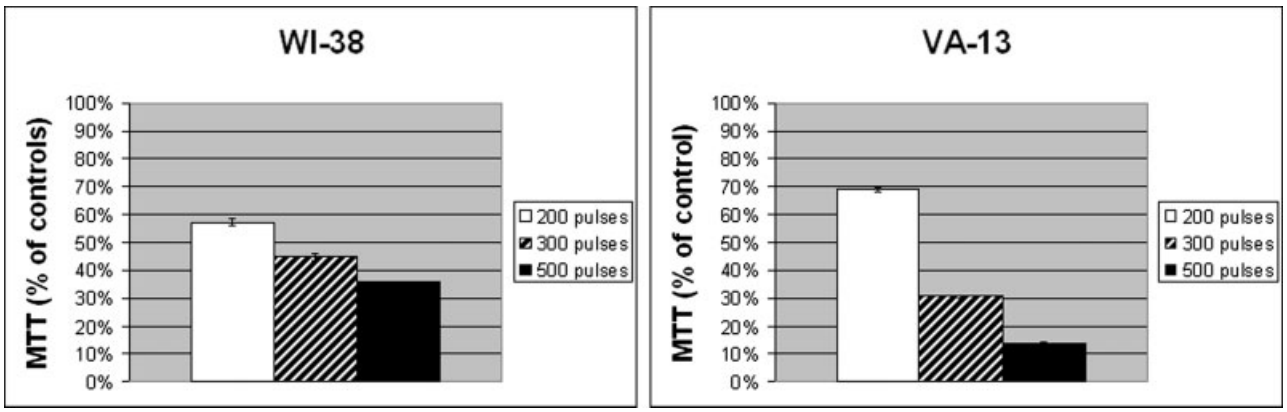

FIGURE 3 – Responses of normal and transformed fibroblasts to pulsing. WI-38 normal human fetal lung fibroblasts and VA-13, matched cells transformed with the SV40 virus, received 200, 300 and 500 pulses of 20 ns duration at 35 kV/cm. Cells were cultured for 96 hr, their numbers were measured by MTT; Results were compared to sham-treated control cells. Results represent the mean and standard error of 9 wells.

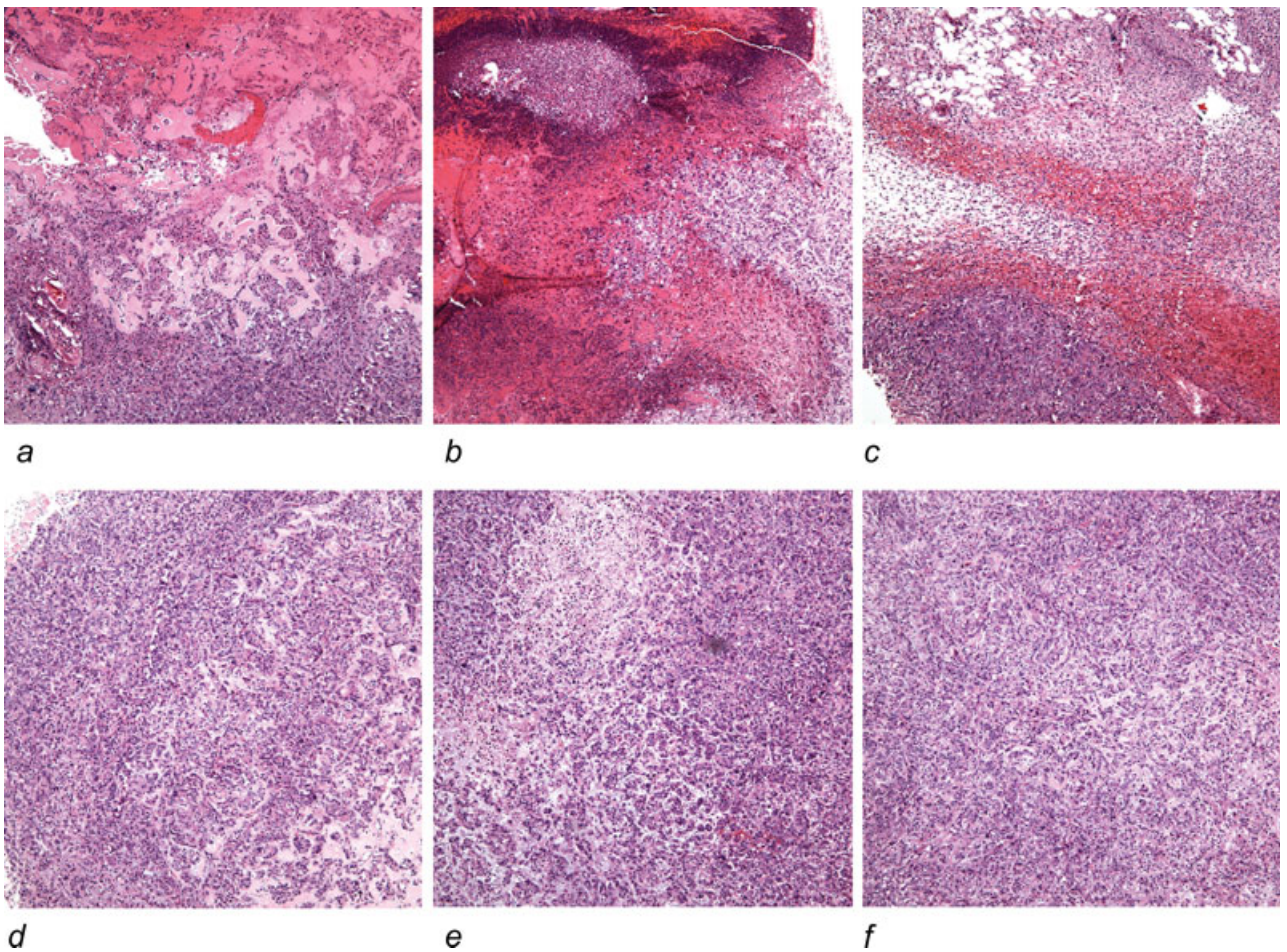

FIGURE 4 – Pathologic evaluation of the pulsed AsPC-1 human pancreatic tumors. Hemorrhagic necrosis was seen by pathologic evaluation of tumors after nanoelectropulses. Shown are tumors pulsed: 65 (*a*), 200 (*b*) and 1000 (*c*) times. For each experimental tumor, the unpulsed, contralateral tumor is shown (Panels *d–f*). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

an unpulsed AsPC-1 tumor on the contralateral flank (Fig. 5*b*). Tumor did eventually recur at the periphery of the pulsed area in each of these complete responding animals. All animals were sacrificed 2 weeks after the initial pulsing. Overlap of tumor weights occurred between pulsed and mock pulsed tumors 2 weeks after pulsing, largely because one tumor in the pulsed group did not have effective pulse delivery on the first day of pulsing secondary to arcing of the electricity. That tumor was almost 10 times larger than the second largest pulsed tumor, skewing the results. When that tumor was removed from analysis, the mean weight of the pulsed tumors was 4-fold less than mock-pulsed tumors (Fig. 5*c*).

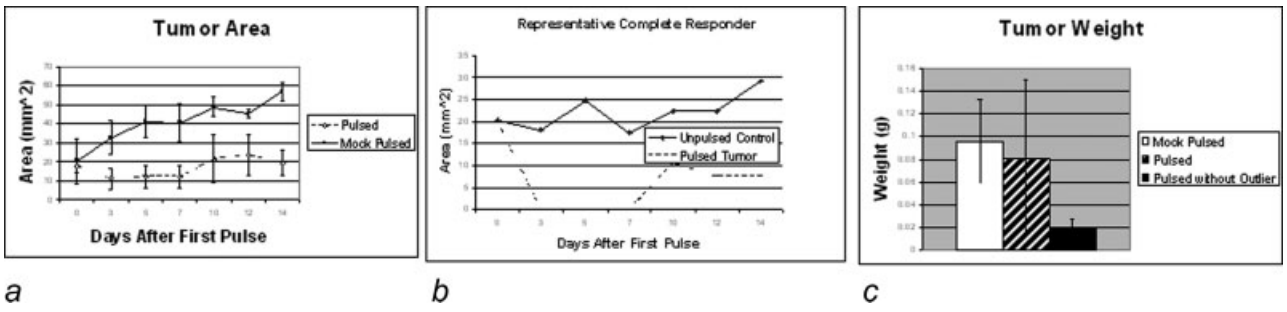

Figure 5 – In vivo experiment evaluating pulsed and mock pulsed tumors. AsPC-1 pancreatic cancer tumors were grown in nude mice. Tumors were pulsed on 3 non-consecutive days during a 5-day period with either 3 bursts of 75 pulses or mock pulses. The area of the tumors was recorded 3 times per week and is shown (*a*). A comparison between a pulsed and unpulsed tumor from one of the mice with a complete response is shown (*b*). Two weeks after the initial pulsing, the weights of the tumors were recorded (*c*).

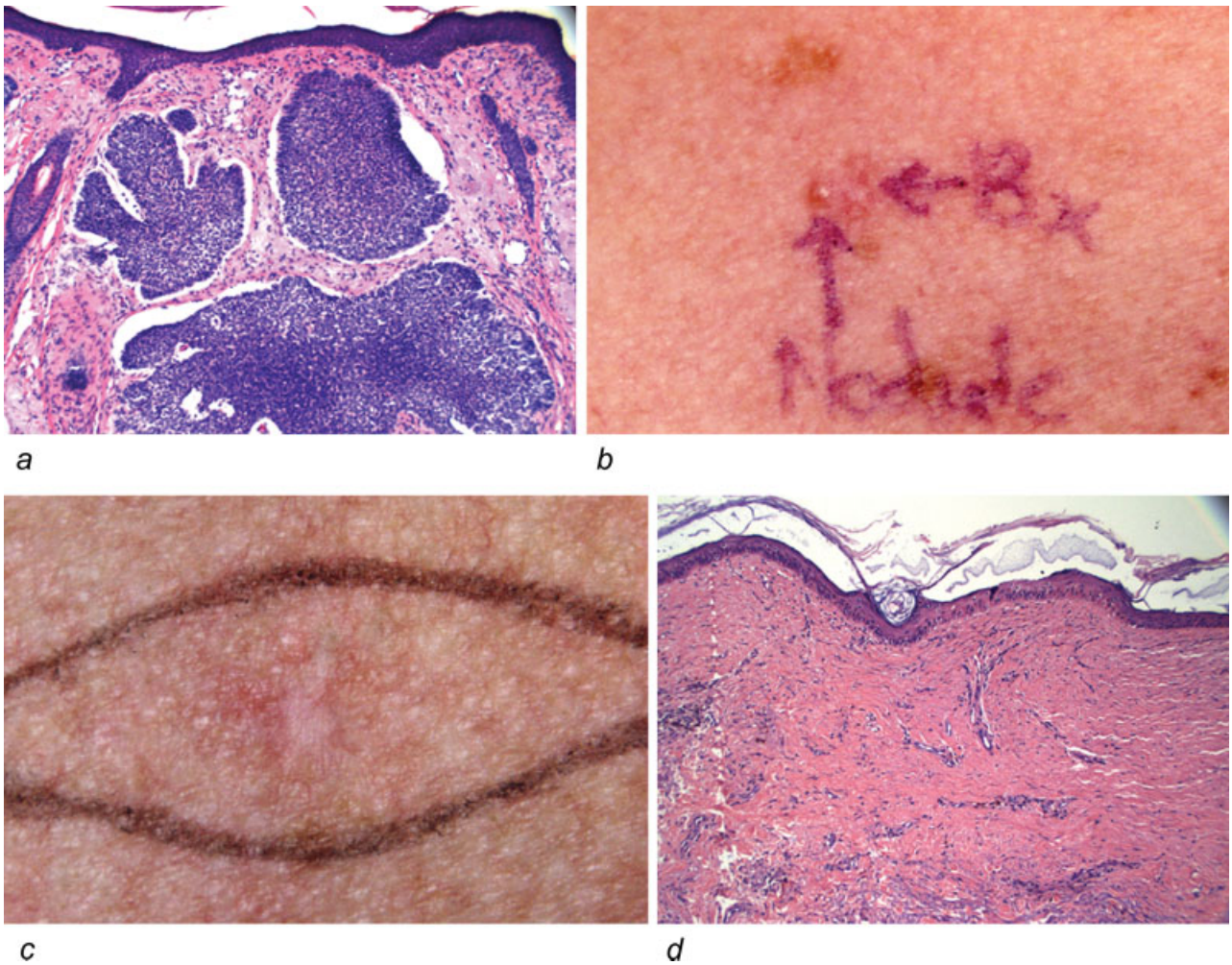

Figure 6 – Case report: basal cell carcinoma treated with nanoelectropulse therapy. An individual was diagnosed with a basal cell carcinoma (*a*). The majority of the basal cell carcinoma remained after biopsy (*b*). Six weeks after receiving nanoelectropulse therapy (200 pulse, 6.5 kV, 20 ns duration), no basal cell carcinoma was visible (*c*). Surgical excision confirmed a complete pathologic response (*d*). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Case report of human basal cell carcinoma response to nanoelectropulse therapy

One of the investigators developed a basal cell carcinoma (Fig. 6*a*). After a diagnostic biopsy, the majority of the basal cell carcinoma remained (Fig. 6*b*). Six weeks after the investigator self-administered 200 pulses to the basal cell carcinoma, clinical resolution was seen with only a small scar remaining (Fig. 6*c*). Surgical excision of the area confirmed a complete pathologic response (Fig. 6*d*).

Discussion

New local therapies are needed in many malignancies. Aggressive local treatment may provide a cure before local complications

arise and metastases occur. Furthermore, while metastatic disease is the most common cause of death among cancer patients, local complications lead to mortality in some patients, and morbidity in many more. As in pancreatic cancer, extensive local spread often prevents surgery and can prevent other therapeutic options. Nanoelectropulse therapy offers a potentially new form of local therapy. Because the effects derive disproportionately from intracellular action, the spectrum of tumors responding, the side effect profile and the degree of response could differ significantly from currently available local therapies.

In this study, we evaluated cell viability of a wide range of malignant cell types *in vitro* in response to nanoelectropulse therapy. Although previous work has characterized biologic responses to nanoelectropulse therapy, cell viability after pulsation has not been rigorously examined in such a wide variety of cancer subtypes prior to this study. The responsiveness to nanoelectropulse therapy of each cell line tested, could not have been predicted based on response to other therapies. The cell death (or decrease in proliferation) was not an immediate response as a result of destruction of the cell membrane, as trypan blue assays immediately after pulsing did not demonstrate significant uptake. This leads us to believe that cell death occurred as result of delayed effects. Traditional poration does not occur in cells treated with nanoelectropulses. Calcium efflux from intracellular stores is seen after such pulses, and calcium release may initiate the apoptotic pathway in pulsed cells, resulting in a cell death that is not caused by immediate destruction of the cell membrane.

Different pulsing regimens led to different responses. The data with Jurkat cells demonstrated that even if energy was held constant by decreasing pulse number to compensate for longer pulses, 20 ns pulses were more effective than 7 ns pulses. In Jurkat cells, higher voltage led to more effective cell killing. Many other cell lines also displayed strong dose effects associated with either more pulses or pulses of higher energy, but other cell lines were not more effectively killed with higher energy regimens. One possibility is that the field strength is not exactly what would be predicted. Textures and shapes of cells vary considerably among cell types, and even among cells of the same type. Dielectric profiles at the cell surface and at critical boundaries in the cell interior are complex and rich in atomic-scale detail. The reported macroscopic mean fields are based on a spherical model, and are only approximations of values at the biomolecular level. Nevertheless, these mean fields are extracted from readily reproducible measurements. Mean fields based on applied pulse voltage, time and energy, allow comparison of experimental conditions among different experiments and different laboratories. Another potential explanation for these differences is that 20 ns pulses, but not 7 ns pulses, reached a threshold for poration of intracellular organelles.

Cell lines displayed significant variability in response to nanoelectropulse therapy. Responses to nanoelectropulses were not cell-type specific, as both pancreatic cancer cell lines and diffuse large B cell lymphoma cell lines showed widely differing responses among cell lines from the same type of malignancy. Possibly, some cell types are more dependent on a particular organelle, like the mitochondria. These cell lines might have greater sensitivity to pulses that are sufficient to lead to poration of that organelle. Results with the WI-38 and VA-13 cell lines demonstrated differences between the normal WI-38 fibroblast cell line and the SV40 transformed WI-38 at some pulsing regimens. SV40 large T-antigen binds and inactivates the p53 and Rb proteins. Perhaps the VA-13 cells cannot undergo G_1 arrest and needed cell repair after the nanoelectropulse injury.

In this work, we sought to demonstrate the efficacy of pulses delivered locally via a catheter in a xenograft model. Pulses less than the charging time constant of the plasma membrane of an isolated cell were studied. Although other *in vitro* work has considered pulses of this duration, *in vivo* work to date has been limited to pulses of much longer duration than those used in our study.[28] In an attempt to minimize possible thermal effects, we limited parameters to 200 pulses at 35 kV/cm *in vitro* and 75 pulse bursts at 6.5 kV *in vivo*. These pulse regimens lead to temperatures below the threshold of 42°C where heat shock proteins are activated.[29] Thus, this study demonstrates that at least the AsPC-1 xenografts of pancreatic cancer cells respond to nanoelectropulses of this duration by a mechanism not involving the traditional heat shock response.

Not all tumors had a complete response after pulsing, and responses were transient. Several technical issues could cause this variability. Arcing happens if the catheter is not correctly applied to the tissue. An arc occurs, for instance, if an air gap is present, as under this condition a local enhancement of the electric field develops. Arcing was not observed, and pulse distortion did not occur when the catheter was properly applied to the tissue. Still, some cases of arcing did occur, and in general, the tumors on these animals grew. One additional technical concern is that tumors are 3 dimensional, and tumor depth cannot be effectively evaluated without invasive monitoring. This was suggested by the only moderate correlation between tumor area and tumor weight. Tumors may have been deeper than anticipated on examination, resulting in residual tumor deeper than the distal extent of the catheter. Future experiments could consider delivery mechanisms that conform to the tumor. This could result in greater efficacy and prevention of tumor recurrence.

The case report in which one of the investigators used the catheter to eradicate his own basal cell carcinoma is interesting and merits further investigation. The parameters used were similar to those that elicited responses in AsPC-1 cells *in vivo*. The disappearance of the basal cell carcinoma was pathologically confirmed, and the cosmetic result was good. Nanoelectropulse therapy with these parameters was well tolerated, with minimal discomfort after topical anesthesia and division of the pulses into 10 pulse bursts. Clinical work will have to proceed in a more orthodox and stepwise fashion accompanied by further work *in vivo*. We plan to explore the clinical utility of this device for basal cell carcinomas. Based on the demonstrated ability of nanoelectropulses to dislocate phosphatidylserine to the external plasma membranes, we feel that combining this modality with immunotherapy is also a potentially promising approach.

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