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Nanoelectroablation of Human Pancreatic Carcinoma in a Murine Xenograft Model without Recurrence

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Abstract

We have identified an effective nanoelectroablation therapy for treating pancreatic carcinoma in a murine xenograft model. This therapy initiates apoptosis in a nonthermal manner by applying low energy electric pulses 100 ns long and 30 kV/cm in amplitude to the tumor. We first identified the minimum pulse number required for complete ablation by treating 30 tumors. We found that the minimum number of pulses required to ablate the tumor with a single treatment is between 250 and 500 pulses. We settled on a single application of either 500 or 1000 pulses to treat pancreatic carcinomas in 19 NIH-III mice. Seventeen of the 19 treated tumors exhibited complete regression without recurrence. Three mice died of unknown causes within 3 months after treatment but 16 lived for 270–302 days at which time we sacrificed them for histological analysis. In the 17 untreated controls, the tumor grew so large that we had to sacrifice all of them within 4 months.

Keywords

nanoelectroablation; nanosecond pulsed electric fields; apoptosis; necrosis; angiogenesis

Introduction

According to the National Cancer Institute, the incidence of pancreatic carcinoma has increased dramatically over the last 40 years in the U.S., Europe and Japan. It is the *fourth* leading cause of cancer-related death in the U.S. (1). Based on estimates from the American Cancer Society, approximately 43,000 people will be diagnosed with pancreatic cancer in the U.S. this year and worldwide over 270,000 people die of pancreatic cancer each year. Fewer than 20% of patients survive the first year and only 4% are alive 5 years after diagnosis (2;3).

Because the pancreas is a delicate, non-regenerative vital organ, it is critical to minimize damage to healthy pancreas tissue when ablating the tumor. Ablation techniques employing hyperthermia have limitations due to variable heat energy dissipation outside of the treatment area as well as the difficulty in monitoring and achieving thermal equilibrium across the ablated lesion (4). A non-thermal and highly localized ablation technique would minimize collateral damage to the healthy tissue in this vital organ. There are four such therapies being tested in clinical trials presently. These include: 1) Electro-chemotherapy in which 100 microsecond long pulses are used to electroporate the plasma membrane to introduce impermeable chemotherapeutic drugs to tumors (5;6); 2) Electro-gene therapy which uses electroporation to deliver genes that locally activate the immune system (7); 3) Irreversible electroporation which uses higher voltage, 100 microsecond pulses to introduce

irreversible pores in the plasma membrane to initiate necrosis (8); and 4) Nanoelectroablation which uses nanosecond pulsed electric fields (nsPEF) to transiently form nanopores in both the plasma membrane and organelle membranes and trigger apoptosis (9). The advantage of nanoelectroablation over the other electroporation-based approaches is that it does not require the addition of any substances around the treated tumor and it induces apoptosis that slowly ablates the tumor over a three-week period and this allows time for the activation of the immune response (10). We have been studying the use of this fourth method for tumor ablation *in vivo* by ablating murine allograft melanoma tumors (11–13).

Towards our goal of applying this therapy to treat human pancreatic carcinomas, it is important to demonstrate that we can ablate these human tumors in a mouse xenograft model without recurrence. Here we used two immunodeficient mouse models, the Nu/Nu and NIH-III strains, to generate pancreatic tumors using two different human pancreatic tumor cell lines. We first determined the minimum pulse number to completely ablate these tumors with a single treatment and then conducted a long-term study demonstrating that these tumors did not recur during at least 270 days following treatment.

Material and Methods

Cell lines

BxPC-3 (14) and Capan-1 (15) human pancreatic cells derived from pancreatic tumors were obtained from ATCC and were cultured in RPMI-1640 and Iscove's modified Delbecco's Medium, respectively, supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) (20% FBS for Capan-1), and 5% Penicillin-Streptomycin (Mediatech). Cultures were maintained in a humidified 37°C/5% CO₂ incubator. When the cultures reached 90% confluence, they were washed once with HBSS (Hank's Buffered Salt Solution), and harvested with trypsin 0.25%/2.2 mM EDTA.

Animals

Thirteen female Nu/Nu and 36 female NIH-III mice (immunodeficient, hairless, albino) from 4–6 weeks old were purchased from Charles River (Boston, MA), and were maintained in the BioElectroMed animal facility. They were provided with 12-hour light/dark cycle at controlled temperature and humidity with food and water *ad libitum*. They were allowed to acclimatize to the laboratory environment for at least one week before experimentation. All procedures were approved by BioElectroMed's Institutional Animal Care and Use Committee.

Tumor Induction

Subcutaneous tumors were made by injecting 10⁶ BxPC-3 or Capan-1 cells in 15 µl of HBSS under the skin using a hypodermic syringe while the mice were under 1.4% isoflurane inhalation anesthesia. One tumor was made on the left flank in each NIH-III mouse and four tumors were made in each Nu/Nu mouse, two on each side, lower and upper flanks. All tumors were allowed to grow for 20–25 days to a size of 4–5 mm in diameter before receiving nsPEF treatment. Transillumination and brightfield photographs of the tumors were initially taken every other day followed by once a week beginning 10 days after treatment.

Nanosecond Pulse Electric Fields (nsPEF) Treatment

We built our own nanosecond pulse generator as described previously (13). It produces 100 ns-long pulses of 15 kV with a rise time of between 10 and 20 ns (fig. 1). When this pulse is applied to a suction electrode with two rows of needles 5 mm apart, a field of 30 kV/cm is

applied to the region between the electrodes. This fast rise time and short pulse duration assured penetration of the electric field into every cell and organelle located between the electrodes (16).

During the entire treatment, the mice were under 1.4% isoflurane inhalation anesthesia and were lying on a warm platform to prevent hypothermia. A drop of sterile petrolatum ophthalmic eye lubricant (Puralube® Vet Ointment, Dectra Veterinary Products, Overland Park, Kansas) was applied to both eyes during anesthesia to prevent dry eyes. A layer of olive oil was applied to the skin surface above the tumor prior to applying the suction electrode so that the space between the electrode and the skin would be filled with oil rather than air. Gases will ionize at the 30 kV/cm field applied and that will lead to an electrical discharge (spark) between the electrodes that could heat the skin. Using a vacuum pump, the tumor was pulled up into the electrode with needles surrounding the tumor. All tumors were treated with a pulsed electric field of 30 kV/cm, 100 ns in duration at a frequency of either 2 or 5 pulses per second in NIH-III or Nu/Nu mice, respectively. In cases where sparking occurred when treating the tumor, the treatment was paused and resumed after additional olive oil was applied and the electrode was repositioned.

Suction electrode

A six-needle suction electrode composed of 2 parallel rows of three needles on each side was used as shown in fig 1. The length of the needles was 1 mm and the spacing between the two rows of needles was 5 mm. Eight vacuum holes are distributed along the grooves surrounding the electrodes to pull the mouse skin onto the electrode.

Design of the *in vivo* study

Determining optimal pulse parameters—BxPC-3 tumors injected into Nu/Nu mice were randomly divided into experimental groups of seven tumors each. Each experimental group was treated with either 250, 500, 1000, 1500 (data not shown), or 2000 pulses. All nsPEF treatments were carried out using 100 ns long pulses, 30 kV/cm in amplitude, with a frequency of 5 pulses per second. The number of tumors in the 250 pulse group was increased to a total of nine tumors in order to verify that 250 pulses were insufficient to eliminate the tumors. The control group consisted of four tumors left untreated and were allowed to grow to a size larger than 4 mm in diameter. Tumor regression/regrowth was monitored by weekly transillumination photography and the final histological analysis of the treated region following sacrifice of each mouse.

Survival after treatment—Thirty-six NIH-III mice were divided into two groups, control and experimental. Each mouse was injected with 10^6 Capan-1 human pancreatic cancer cells on the left flank region. The tumors in 19 mice were treated with either 500 or 1000 pulses (30 kV/cm, 100 ns) when the tumor grew to 4–5 mm in diameter. The remaining 17 mice were used as controls and were sacrificed when their tumors reached 1500 mm³. The tumors were observed weekly by transillumination and brightfield photography during the 270 days post treatment. Following sacrifice of the animals, all treated regions were removed for histology unless stated otherwise.

Tumor observation

Transillumination and brightfield photographs of the tumors were initially taken every other day after treatment, and beginning 10 days after treatment photos were taken once per week. The tumors were considered eliminated when they could no longer be detected under transillumination.

Histology

Mice were sacrificed at various times after treatment or when the tumor size exceeded 1500 mm³. The treated regions were removed, laid flat on a surface to prevent curling of the specimen, and fixed in 10% buffered formalin. The specimens were embedded in paraffin and sectioned for histology. Some were serial sectioned at 100 µm intervals across the entire region.

Results

Optimizing pulse number

Past studies of nanoelectroablation on murine melanomas and basal cell carcinomas indicated that the minimum effective field strength exhibiting high efficacy is 30 kV/cm. Using this amplitude and our fixed pulse duration of 100 ns, we treated subdermal BxPC-3 pancreatic carcinoma tumors with a range of pulse numbers at 5 pulses per second. Seven tumors each were treated with 250, 500, 1000, 1500 (data not shown) or 2000 pulses. All tumors treated with 2000, 1500, 1000, and 500 pulses were completely ablated within 3 weeks. Five out of seven of the tumors treated with 250 pulses were eliminated; two additional BxPC-3 tumors were treated to confirm the recurrence of tumors following this treatment (fig. 2). The two additional tumors were not eliminated completely and the tumors recurred, suggesting that a pulse number between 250 and 500 could possibly eliminate the tumors completely. However, we decided to use 500 and 1000 pulses to eliminate Capan-1 tumors completely with a single treatment in our long-term study.

Nanoelectroablation Response

Nanoelectroablation of both BxPC-3 and Capan-1 pancreatic tumors results in epidermal necrosis (scab formation) within 3 days after treatment (fig. 2). Over the following 1–2 weeks, the surface scab shrinks and is eventually rubbed off by the host. This typically takes 7 days following a short treatment of fewer than 500 pulses and up to 14 days after treatments with more than 500 pulses. The treated tumor is located beneath the dermis and is much deeper in the skin than the scab. It also shrinks steadily and usually is no longer visible in transillumination by 3 weeks after treatment. Brightfield and transillumination images were taken weekly thereafter and evaluated to monitor possible tumor recurrence. Newly regenerated skin is present where the scab had been located and this new skin appears lighter than the original skin.

Survival after Treatment

Once we determined the optimal pulse number to ablate these pancreatic carcinomas, we designed a long-term study to detect any tumor recurrence. A single Capan-1 pancreatic tumor was induced on the left side of 36 NIH-III mice. We treated the tumors in 12 of the mice with 500 pulses, 4 with 1000 pulses and 1 each with 267, 637 and 867 pulses, respectively. These odd pulse numbers were a result of equipment failures. These pulses were 30 kV/cm in amplitude and 100 ns long, administered at 2 pulses per second when the tumors were 4 to 5 mm in diameter (fig. 3). Seventeen out of 19 nanoelectroablated tumors showed complete regression. The treated site was photographed over the course of at least 300 days and appeared tumor-free within 3 weeks. The animal was then euthanized and the treated region was removed for histological analysis. The remaining two tumors were in two of the mice that died of unknown causes before the tumor completely regressed. The third mouse that died showed complete tumor regression.

The remaining 17 mice served as untreated controls and were sacrificed when the tumor reached 1.5 cm in diameter (1500 mm³). All controls had to be sacrificed within 4 months due to their enlarged tumor size (fig. 4).

A histological analysis of the treated region was conducted on all 17 of the experimental mice. None of the histological sections had any pancreatic tumor remnants. For three of them we analyzed serial sections taken every 100 μm . No residual pancreatic tumor cells were detected in any of these sections (fig. 5). All histological evidence suggests that nanoelectroablation completely ablated these pancreatic tumors and the only changes in the treated skin region noted many days after the scab fell off was a reduction in the local pigment concentration and hair follicles as can be seen in figs. 3 and 5.

Discussion

New Findings

This is the first demonstration that 100 ns pulsed electric fields can eliminate human pancreatic carcinomas in a xenograft murine model system. It is also the first long-term study indicating that these tumors do not recur for at least 270 days after being nanoelectroablated. These findings support our contention that nanoelectroablation can be used to treat human pancreatic cancer *in situ* if the electrodes can be positioned around the tumor using an imaging technique such as ultrasound.

Optimal Pulse Parameters

Previous work from our group treating murine allograft skin tumors indicated that 2000 pulses of 100 ns and 30 kV/cm were required to ablate murine melanomas. The finding here that only 500 pulses are needed to nanoelectroablate human pancreatic carcinomas in the murine model sharply reduces the required treatment time and provides a basis on which to design a human pancreatic cancer clinical trial. Toward this goal, we are developing electrodes to treat pancreatic cancer in conjunction with an echoendoscope imaging system.

Side Effects

The histology of the treated skin regions indicated that the regenerated skin lacked hair follicles and exhibited some fibrosis. These Nu/Nu and NIH III mice are immunodeficient and hairless so the lack of hair follicles in their regenerated skin is expected. When we treat mice that have the normal hair density, we observe hair in the regenerated skin so this apparent side effect is probably due to the mouse's genetics rather than a side effect of nanoelectroablation. When we examine healthy tissue adjacent to treated skin regions, we find no abnormalities.

Nanoelectroablation results from other groups

Gundersen's group at the University of Southern California has developed a 20 nanosecond pulse generator and they have used it to nanoelectroablate the AsPC-1 human pancreatic tumors in a xenograft nude mouse system with 20 ns long pulses. They reported a substantial reduction in tumor mass within 2 weeks after three treatments of 225 pulses each (17). In that same paper they also reported the successful nanoelectroablation of a human basal cell carcinoma.

The Center for Bioelectronics at Old Dominion University has reported the successful nanoelectroablation of liver tumors in a murine allograft model using 100 pulses of 100 ns duration and 55 kV/cm (18). This group has also demonstrated the initiation of caspase activity in melanoma tumors treated *in vivo* that peaks at 6 h and DNA fragmentation peaking at 3 h after nsPEF treatment (19).

The mechanism of nanoelectroablation

Over the past decade, several groups have been investigating the cellular responses to nsPEF application. The earliest of these is the formation of nanopores in the plasma membrane (20–23) and intracellular membranes (24–26) followed by a rapid change in membrane potential (27) and a transient increase in intracellular Ca^{2+} (12;28–30). Additional early events include phosphatidylserine externalization (31) and DNA fragmentation (12;32). The Ca^{2+} increase may be triggering other observed changes such as the transient phosphorylation of multiple proteins in the MAPK pathways (33;34), the generation of reactive oxygen species (35) and apoptosis (19;32;36;37). However, Beebe's group has presented some data that suggest the nsPEF-triggered Ca^{2+} increase is not required for caspase activation (38). More work is needed to identify the detailed sequence in the signal transduction cascade connecting nanopore formation and apoptosis initiation. However, a very interesting hypothesis has recently been proposed by Weaver's group that involves a two-step process: 1) The intracellular Ca^{2+} increase leads to a mitochondrial Ca^{2+} increase; 2) This increased mitochondrial Ca^{2+} causes a swelling of the outer mitochondrial membrane that eventually ruptures it (39;40). This would release cytochrome C and other death molecules and could explain the requirement of hundreds of pulses to trigger apoptosis.

Conclusion

We find that applying 500 pulses 100 ns long and 30 kV/cm in amplitude can ablate human pancreatic tumors in a mouse xenograft model with a single treatment without recurrence. This suggests that this nanoelectroablation therapy should be effective in the treatment of human pancreatic carcinomas when we are able to deliver these pulses to the tumors *in situ*. We are presently developing delivery systems that can accomplish that in conjunction with an echoendoscope or laparoscope.

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Novelty and Impact

This is the first paper to determine the minimum pulse number required to nanoelectroablate human pancreatic carcinomas and to demonstrate that the ablated tumors do not recur for at least 270 days. This demonstrated efficacy of this new non-thermal therapy is a breakthrough that could lead to a much more effective therapy for human pancreatic carcinoma. Current ablation therapies such as radio frequency ablation use hyperthermia to ablate and the high tissue heat conduction leads to thermal damage of healthy pancreas tissue surrounding the tumor. Nanoelectroablation precisely targets only tissue between the electrodes to minimize collateral damage.

Figure 1.

Oscilloscope recording showing a typical voltage pulse (above) and current delivered (below) to the tumor through the electrode shown in the inset photo. Note the groove surrounding each needle where suction is applied to pull skin against the electrode allowing it to penetrate into the dermis. Each row of three electrodes was at the same potential and 15 kV was applied between the two rows spaced 5 mm apart.

Figure 2.

Human pancreatic tumor regression in Nu/Nu mice after nanoelectroablation. BxPC-3 tumors were treated with nsPEF 100 ns long, 30 kV/cm, at a frequency of 5 pulses per second. The number of pulses used in each treatment is indicated at the top of each column. Each column shows the brightfield and transillumination images taken on the day after treatment indicated by the number at the far left of each row. Scale bar on the first brightfield photos applies to all photos. (–) means before treatment. *Far right column presents a tumor that is not fully eliminated after receiving a single treatment of 250 pulses as reoccurrence is seen on day 21; the tumor was retreated on day 28. BxPC-3 tumors began to scab 1 day after the treatment. The scabs remained attached to the skin for about 14 days. The presence of the scab made it difficult to pull the skin over onto the light post for taking transillumination photos. Slight edema occurred during the recovering period as reflected by the red-yellow pigment seen on the transillumination photos.

Figure 3.

Bright field and transillumination images of selected Capan-1 pancreatic tumors growing in NIH-III mice. On the left, an untreated tumor has grown to 1.5 cm in diameter over 55 days after injection and the mouse was then sacrificed. The two tumors on the right were treated with 500 pulses 100 ns long, with an amplitude of 30 kV/cm, at 2 pulses per second on day 0 after the top photos were taken. The number of days after nanoelectroablation on which each pair of photos was taken is indicated on the upper right of the transillumination photo. Both tumors were completely ablated and did not recur up to 302 days after nanoelectroablation. The scale bar in the first brightfield image applies to all the photos in this figure.

Figure 4.

Survival curve for both mice with nanoelectroablated human pancreatic tumors as well as untreated controls. Capan-1 pancreatic tumors were induced in these mice on day 0 and treated with 500–1000 pulses (30 kV/cm, 100 ns) when they were 4 mm in diameter. Treated mice were sacrificed at 300–330 days after tumor injection to conduct histology on treated skin regions.

Figure 5.

Three photomicrographs of the same histological section taken at different magnifications through a treated skin region fixed 279 days after nanoelectroablation with 500 pulses (30 kV/cm, 100 ns). No residual pancreatic carcinoma was detected. Some notable characteristics are the absence of hair follicles and a mild lymphocytic (arrow 1) infiltrate in the dermis. Fibrosis is indicated by the parallel orientation of fibers in the dermis (arrow 3) and there is no increase in vascularization. Other infiltrates are macrophages (arrow 2) and fibroblasts (arrow 4). The hypodermis is thinner than in untreated skin. The asterisk in the top two images indicates the region that was magnified in the image directly below.