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Treatment of breast cancer through the application of irreversible electroporation using a novel minimally invasive single needle electrode

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Abstract

Irreversible electroporation (IRE) is a therapeutic technology for the ablation of soft tissues using electrodes to deliver intense but short electric pulses across a cell membrane, creating nanopores that lead to cell death. This phenomenon only affects the cell membrane, leaving the extracellular matrix and sensitive structures intact, making it a promising technique for the treatment many types of tumors. In this paper, we present the first in vivo study to achieve tumor regression using a translatable, clinically relevant single needle electrode for treatment administration. Numerical models of the electric field distribution for the protocol used suggest that a 1000 V/cm field threshold is sufficient to treat a tumor, and that the electric field distribution will slightly decrease if the same protocol were used on a tumor deep seated within a human breast. Tumor regression was observed in 5 out of 7 MDA-MB231 human mammary tumors orthotopically implanted in female Nu/Nu mice, with continued growth in controls.

Keywords

Tumor ablation; IRE; N-TIRE; Minimally invasive surgery; Electroporation; Focal orthotopic tumor therapy; Translational protocols

Introduction

Therapeutic electroporation, a technique that increases the permeability of cell membranes by exposing the cell to electric pulses [25], is becoming increasingly popular as a minimally invasive surgical technique for introducing small drugs and macromolecules into cells within specific areas of the body [17]. The external electric field to which the tissue is exposed affects the transmembrane potential. Depending upon the change in potential, the electroporation pulse can either have no effect on the cell membrane, reversibly open the cell membrane, after which cells survive, or irreversibly open the cell membrane, after which the cells die in a process known as irreversible electroporation (IRE) [17].

Irreversible electroporation is a promising new technology to treat pathologic tissues, such as tumors. Typical procedures deliver short-length, high voltage electric pulses using needle electrodes placed in or around the targeted region [25], making the treatment minimally invasive and localized. Recent studies in animal models have demonstrated that substantial volumes of tissue and cutaneous tumors may be ablated in a non-thermal manner using IRE [1,4]. However, articles using IRE for the treatment of solid tumors in vivo is currently limited to a single study [1], and its potential for the treatment of breast cancer has not been explored in vivo.

Current clinical therapy for breast cancer most often includes a combined approach of surgical resection via mastectomy or lumpectomy, irradiation, and adjuvant chemotherapy [10]. Although great strides have been made in breast conserving therapy, the invasiveness of surgical resection often results in significant scarring and disfigurement [8,10]. Intramammary scar tissue may form spiculated lesions that resemble various benign or malignant entities when viewed mammographically [11]. This scarring complicates monitoring for residual tumors and cancer recurrence, often requiring ultrasound, clinical examination, and/or magnetic resonance mammography, which may be inaccessible and expensive, and often a biopsy is still required to determine the true nature of these lesions [11,20].

Since breast conserving therapies that use lumpectomy have shown equivalent success relative to mastectomy [9], there is an incentive for minimally invasive focal ablation techniques that can reduce scarring and improve healing after treatment. IREs mechanism means that it is unaffected by blood flow, and the electric fields applied to induce IRE affects only the cell membrane, sparing the extracellular matrix and sensitive structures such as myelin sheaths and major tissue vasculature, allowing for rapid lesion resolution and minimal scarring of the treated volume [7,19,21]. Due to immediate changes in the treated tissue's permeability, the affected regions may be monitored in real-time using ultrasound [21]. There is a high resolution demarcation between treated and normal regions [7], and it has been shown that the heterogeneous properties of certain structures, such as lactiferous ducts, may protect them from electroporation pulses, preventing damage and allowing for continued functioning [2]. Electroporation treatments promote an immune response [17,19], including areas experiencing reversible electroporation [17]. Furthermore, electric fields may be predicted through numerical modeling [4,6], allowing for reliable treatment planning.

We hypothesize that thorough tumor ablation may be achieved by IRE-sufficient electric fields distributed by an electrode capable of clinical application. For IRE to be an advantageous alternative to surgical resection, it must be shown that tumors can be treated by IRE with minimal effects to the surrounding healthy tissue. Additionally, current in vivo investigations using IRE to treat tumors used plate electrodes placed on either side of the exposed cutaneous tumor. Therefore, employing IRE as an improved focal ablation technique requires the design and use of an optimized, clinically relevant electrode to minimize invasiveness and facilitate simple treatment implementation. For breast carcinomas, treatment may be facilitated through a small single needle electrode, similar to ones used in taking a biopsy, which may be easily placed under ultrasound guidance.

In this article, we show that IRE may be a suitable candidate for minimally invasive treatment of breast tumors. This was done by treating orthotopically implanted human mammary carcinomas in an in vivo mouse model using a novel electrode design. To the best of our knowledge, this is the first study to treat tumors with IRE that were grown in a physiologically relevant location. Furthermore, this is the first study to treat tumors using an electrode capable of being easily translated to and implemented in a clinical setting.

Materials and methods

Electrode design

Prior to treating the tumors, different electrode styles were considered to select an appropriate compromise between treatment effectiveness and procedural robustness. Multiple electrodes would enlarge the electric field throughout the tumor, but would increase placement complexity, increasing chances for human error and physical damage from electrode penetration. Procedural practicality was vital to developing translational treatment protocols, and thus a single needle bipolar electrode design was used. The electrode prototype developed uses an insulating body for penetration into deep tissues with the tip containing two electrically conductive surfaces separated by an additional insulating layer. These conductive surfaces are the anode and cathode to deliver the energy of the IRE pulses. This design was chosen because it is capable of treating the tumors without requiring excessive invasiveness or complex electrode arrangements and placement. The dimensions of the needle used in the experiments are shown in Fig. 1a. The electrode lengths were selected to fit all four layers of the electrode within an 8-mm long tumor. The diameters were selected to achieve a sufficient electric field distribution while keeping the 1.5 mm largest layer narrower than the 1.7 mm of typical probes used in cryoablation [16].

Numerical Model of Electric Field

Two three-dimensional finite element numerical models were created (COMSOL, Stockholm, Sweden) to gain an understanding of the behavior of the electric field distribution when 1300 V was applied to one charged surface and the other was set to ground, as was done in the animal studies. The models used the dimensions of the needle, shown in Fig. 1a, and the dimensions of the treated tumors. The electrical properties of the tissue were chosen to simulate a conductive breast tumor ($\sigma_t = 0.25$ S/m [24]) within a layer of fatty peripheral tissue ($\sigma_p = 0.02$ S/m [24]). The first model simulates the treatments done on the animals, where the system was placed over an anisotropic layer of muscle ($\sigma_{m,perpendicular} = 0.055$ S/m and $\sigma_{m,parallel} = 0.8$ S/m [5]), a schematic of this setup may be seen in Fig. 1b. The second model used a large outer domain of peripheral tissue to understand the electric field distribution if the protocol used was performed deep within a human breast.

Animals studies

Female Nu/Nu mice (Crl:NU-Foxn1^{Nu}) were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed in individually ventilated cages in groups of five under specific pathogen free conditions, and were allowed access to food and water ad libitum. Mice were approximately 6 months old at the time of tumor implantation.

For tumor implantation, luciferase expressing human breast carcinoma cells, MDA-MB231-luc(D3-H1) (Caliper Life Sciences, Hopkinton, MA), were grown to log phase in 15 cm tissue culture dishes (Corning Costar, Lowell, MA) in MEM supplemented with 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine (all from Invitrogen, Carlsbad, CA). Cells were grown from frozen stocks prepared after three passages of the initial cell shipment from Caliper (formerly Xenogen), and were amplified for three passages prior to use for tumor implantation. Cells were treated with trypsin (Invitrogen) then washed in phosphate buffered saline (PBS) (Invitrogen). Tumor cells were resuspended at a concentration of 2×10^7 cells/ml in an ice cold mixture of 50% Matrigel (BD Biosciences, San Jose, CA)/50% PBS, and 100 μ l of the suspension was injected into the fourth inguinal mammary fat pad of 11 mice.

Tumor growth was measured over time using calipers. Volumes of the tumors were calculated according to the equation: $(1 \times w^2)/2$. Tumors were treated when the two largest perpendicular diameters reached 5–8 mm, since it was predicted that in the near future, 50% of newly diagnosed breast cancers will be <1 cm [2]. The experiment was conducted using a total of 11 mice divided into two groups, treated on separate days. In the first group of three mice, two were treated by IRE and the third served as a control. In this group, tumor growth was measured over time using calipers alone until the time of killing. In the second group, five mice were treated with IRE and three served as controls. Tumor growth was measured by calipers and by bioluminescent imaging, as described below. At the time of killing, the tumor site for this group was excised and prepared for histology as described below.

To perform the treatment, the mice were anesthetized by isoflurane inhalation (mixed with oxygen and administered to animals at 3% for induction and 1–2% for maintenance). The skin over the tumor was disinfected by scrubbing with an iodine surgical scrub and then rinsed with 70% isopropyl alcohol. After preparation of the surgical site, a small (approximately 5 mm) incision was made to allow for visualization of the tumor and easy placement of the electrode. Next, the electrode was inserted into the tumor, parallel to the mouse's body, through the incision and advanced into the center of the tumor. Seven tumor-bearing mice were treated with 100 pulses, each 100 μ s long, in 3 s intervals. After 25 pulses, the polarities of the electrode were reversed and a further 25 pulses were administered. This process was repeated until 100 pulses were reached. Initially, the applied voltage was set at 1300 V. Due to changes in tissue conductivity that occurred during treatment, the voltage was reduced in 100 V increments at the first sign of electrical arcing between the anode and cathode of the electrode. The minimum voltage reached was 1100 V. Following treatment, the incision was closed using 5-0 nylon sutures (Ethicon, Cornelia, GA) and mice were injected subcutaneously with ketoprofen (5 mg/kg). Sutures were removed 5 days later. Control mice ($n = 4$) were treated in a similar manner, without any applied voltage. All mice were followed for 4 weeks after treatment, at which point the study was ended.

Tumor growth was monitored both by calipers and by bioluminescent imaging for 30 days. For imaging analysis, mice were injected intraperitoneally with 75 mg/kg D-Luciferin (Xenogen, Alameda, CA) in PBS. Bioluminescence images of anesthetized mice (isoflurane inhalation) were acquired by using the IVIS Lumina II Imaging System (Xenogen) 5–10 min after injection. Exposure settings were determined using the Auto

feature of the instrument. Analysis was performed with LivingImage software (Xenogen) by quantifying photon flux (measured in photons/s/cm²/steradian). Images were set at the indicated pseudocolor scale to show relative bioluminescent changes over time.

Histology

The tumors and adjacent tissue were completely excised and preserved in 10% neutral buffered formalin for at least 24 h. After trimming, the tissues were embedded in paraffin and processed routinely for histology, cut at 4–6 µm and stained with hematoxylin and eosin. Images were taken using a Zeiss Axioplan 2 microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) and a digital camera at 10× magnification. Digital images were imported into Adobe Photoshop, but were left unmodified other than cropping and digitally zooming to emphasize specific regions.

Results and discussion

All mice tolerated the treatment well. Following treatment mice were monitored for signs of pain or distress as indicated by: changes in posture (hunched or sleeping appearance), piloerection, dehydration, changes in behavior (increased timidity or aggression, isolation), changes in activity (reflex withdrawal, biting at the treated area), and changes in locomotion (unsteady gait, lameness). After recovering from anesthesia, no signs of pain were noted for the duration of the study. Growth and regression of the tumors was normalized to their initial volume by dividing their volume, V , by their volume at the time of treatment, V_0 . The relative growth curves of all tumors may be found in Fig. 2, where the relative volumes of the individual tumors are shown versus time.

The bioluminescent images seen in Fig. 3 are from the second experimental trial and show that the fully regressed physical tumors contained no sign of viable cancerous cells at 4 weeks post-treatment. The tumors from the treatment group that did not regress showed continued growth, though no evidence of tumor metastasis was observed.

Histological examination of the tissue taken from the site of tumor implantation was done in a blinded fashion by a board certified anatomic veterinary pathologist (NDK). Three control tumors and five IRE-treated tumors, three of which regressed, were examined. As shown in Fig. 4, the tumors from the control group had central necrotic cores surrounded by sheets of viable tumor cells, among which many mitoses were noted. Thick bands of plasma cells, and fewer lymphocytes, were present along the outer margins, interpreted as host reaction to the tumor (Fig. 4a). The two IRE-treated tumors that did not regress had a similar appearance (not shown). For the three tumors that regressed after treatment, the neoplastic cells were absent (Fig. 4b), apart from one of the three cases, where a few cells (mostly degenerate) were present (Fig. 4c). Mitoses were not noted in this lesion and the interpretation was that the tumor was near the final stage of regression. Reactive fibroplasia and neovascularization, with lymphoplasmacytic infiltration were present in the dermis and subcutis along the margins of the lesions.

By compiling the data from the tumor growth as measured by calipers, bioluminescent imaging of tagged cancer cells from the tumor, and histological examination, it may be determined that tumor regression occurred for five out of the seven treated tumors (both treated tumors from the first group, and three out of five from the second group). All control tumors showed continued growth over the experimental period. Analysis of the tumor growth from caliper measurements (continued growth against regression) was performed using Pearson's test with JMP software (JMP, Cary, NC), where it was found that treatment is statistically significant in regressing the tumors ($P = 0.0221$).

The numerical models of electric field distribution may be seen in Fig. 1c, d, where each color represents an electric isofield contour, and the volume within each color is at least that respective electric field. The black borders represent the physical domains in the models, including the tumor and all peripheral tissues. The distribution from the treatment around the average tumor dimensions may be seen in Fig. 1c, where it appears that the tumor was entirely covered by an electric field of approximately 1000 V/cm. This suggests that this electric field may be treated as the minimum threshold required to induce IRE in these tumors in vivo. This finding is consistent with previous in vitro experiments that used a similar protocol on the same cell line while in suspension and determined the electric field threshold to be 1000 V/cm [18]. The second numerical model output of Fig. 1d shows how the electric field distribution would change in a clinical setting, where the tumor would likely be located deep within the breast. The results indicate that in deep tumors, the applied voltage should be slightly increased relative to that used in the mouse model to ensure complete treatment of a similarly sized tumor.

Typical surgical resection procedures remove a 0.5 cm margin around the tumor to ensure removal of any potentially infiltrative cells beyond the tumor borders [8]. Incorporating treatment margins would likely be employed in IRE ablation therapies as well by expanding the electric field so that the IRE threshold found to be 1000 V/cm from the numerical model in Fig. 1c reaches 0.5 cm beyond the tumor. Conventional focal ablation therapies, such as radiofrequency (RF) ablation and cryosurgery, rely on thermal energy [15,22,23], which present complications including inconsistencies between the predicted or visualized heated/cooled zone and true cell death regions [14,22] and trouble with thermal dissipative properties (the blood perfusion “heat sink” or “cold sink” effect) of vascularized tissue [13,15]. Additionally, hyperthermic techniques such as RF ablation can induce charring at the electrode interface [15], require minimum treatment depth of at least 1 cm to prevent skin injury [23], and produce significant scar tissue [14], reducing accurate follow-up. IREs benefits, including rapid lesion creation and resolution, as well as minimal scarring [19,21], means that larger treatment regions including a margin would likely have fewer negatives than conventional surgical and ablative therapies.

Expanding and shaping the electric field to treat larger tumors and margins beyond the tumor may be done using several techniques to ensure complete treatment of the targeted area while minimizing effects on healthy tissue. One method includes using customized electrode geometries, something easily done with the electrode design used. For reasonably simple geometries, such as spheres and ellipsoids, the layer diameters and lengths of the electrode could be altered. For instance, longer and further separated cathodes and anodes will axially extend the treatment margins, while shortening them will produce a more spherical distribution. The diameters may be made larger to increase treatment volume, or narrower to reduce invasiveness or improve insertion for stiffer tumors that thicker needles may have trouble penetrating.

For very large or highly irregular lesion geometries that cannot reasonably be treated with a single insertion of the electrode, multiple insertions of electrodes allow treatment volume to be more effectively shaped, as is often done with thermal focal ablation therapies [16,23]. When applying combinations of treatments, each pulse application will have its own electric field distribution and thus region of IRE cell death, which may be superimposed. In this way, each portion of the tumor may be independently treated until the entire mass has been ablated. The electrodes used for such an approach may be very small or of a complex geometry to keep invasiveness low. This versatility can facilitate the treatment of many different cancers located throughout the body while minimizing secondary effects from physical invasiveness. Additional analysis of electrode selection and treatment planning to shape lesions may be found in [3,12,18]. Furthermore, electric pulse parameters may be

adjusted to treat different volumes. Because IRE lesions follow the electric field strength, larger volumes may be simply treated by increasing the applied voltage.

Most previous in vivo investigations of IRE used healthy tissue, and do not account for the potential differences in IRE related to the structure and organization of cancerous tissue [7,19]. Previously published studies using IRE to treat tumors used plate electrodes placed on the tumor to deliver IRE pulses [1]. This required that the entire tumor be physically exposed to allow for electrode placement. We present the first investigation to treat solid tumors with a clinically relevant electrode.

Understanding why two tumors continued to grow provides insight into improving upon the findings of this initial study. Both tumors that did not regress were the widest, one of which being the largest (9.7×8.1 mm); suggesting that the size or shape of the tumor may have exceeded the treatment region for the protocol used. This is supported by the bioluminescent image of the largest tumor taken 3 days after treatment (not shown), showing no living cancer cells near where the electrode was placed, but some remained at the outer edges of the tumor, where the electric field would be the weakest. In the image 4 weeks after treatment (Fig. 3b), the largest tumor is the one furthest to the right, and the greatest concentrations of cancer cells are in these same regions, suggesting that the surviving cells were responsible for the continued growth of the tumor. To prevent this in future studies and treatments, a maximum treatment region should be found for a given protocol, such as through numerical modeling, and the treatment region should be adjusted as previously discussed for targeted volumes larger than this maximum.

In addition to preventing the errors that may have led to the two tumors that did not regress, the findings of this study can be improved in future studies and treatments. This experiment used a standardized electrode geometry and protocol, which did not allow for variations in tumor size or shape. In clinical settings, the electrode dimensions and protocol used may be adjusted according to treatment demands. Furthermore, tumor response was obtained in immunodepressed mice without the combinatorial approach typical of breast conserving therapies. Additional treatment modalities, such as electrochemotherapy [17], can selectively kill cancer cells experiencing electric fields above the reversible electroporation threshold (~ 250 V/cm). This as well as an immune response, which is promoted by electroporation [17,19], will improve the effectiveness and reliability of these treatments by killing cancer cells which may otherwise survive IRE alone.

Conclusion

This study found that human breast cancer tumors orthotopically implanted in the mouse mammary fat pad can be successfully treated using IRE delivered by a novel, clinically applicable IRE electrode design. These findings suggest that IRE could be an advantageous alternative to surgical resection for breast conserving therapy.

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Fig. 1.

Electrode design and numerical treatment models. **a** Electrode and dimensions used for treatments. **b** Schematic of model setup showing the electrode inserted into the tumor surrounded by fat and sitting on top of a layer of muscle, similar to the experiments performed. **c** Numerical models of electric field output from experiments, where each layer corresponds to electric isofield contours. The tumor is entirely within the 1000 V/cm contour, suggesting a reasonable threshold to use for treatment planning. **d** The electric field distribution if same procedure were applied deep within human breast tissue

Fig. 2.

Tumor growth curves. The growth of individual tumors over time is presented relative to the volume measured immediately prior to treatment. Closed circles represent tumors in mice that received IRE treatment while open circles represent the tumors in mice receiving only a mock treatment. Continued growth can be seen in all control tumors and two of the treatment tumors while all other treated tumors regressed

Fig. 3. Bioluminescence of IRE-treated tumors: The five mice of the second IRE treatment group with bioluminescence from the tagged cancerous cells from the tumors may be seen in location and density. The time the images were taken were at **a** 1 h prior to treatment, where all mice have tumors of approximately the same size, and **b** 4 weeks post-treatment, where it can be seen that there is no trace of cancerous cells in the first 3 mice, while the two that did not regress continued to grow and spread

Fig. 4. Histology of the mammary fat pad of tumor-bearing mice 30 days following IRE treatment. **a** Mock treatment showing densely packed, viable cancer cells in the subcutis and dense inflammatory cell infiltrate along the margin. *Inset*: higher magnification of the neoplastic cells with mitoses visible (*arrow*). **b** IRE treatment showing tumor regression with only remaining stromal cells, homogeneous eosinophilic material and adipose tissue (clear cells). *Insert*: Higher magnification of the central IRE-treated region. **c** IRE treatment showing near-complete tumor regression. *Inset*: higher magnification of the few atypical cells (*arrow*) remaining. All images were taken at 10× magnification. *Insets* are digital zooms (40× total magnification) taken from the corresponding low power field. *Scale bars* represent 200 μm