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Irreversible Electroporation Is a Surgical Ablation Technique that Enhances Gene Transfer

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Abstract

BACKGROUND—Reversible electroporation has long been used to transfer macromolecules into target cells in the laboratory by using an electric field to induce transient membrane permeability. Recently, the electric field has been modulated to produce permanent membrane permeability and cell death. This novel technique, irreversible electroporation (IRE), is being developed for nonthermal cancer ablation. We hypothesize that outside the central zone of IRE exists a peripheral zone of reversible electroporation where gene transfer may occur.

METHODS—IRE of the liver was performed in a Yorkshire pig model with administration of a plasmid expressing the marker gene green fluorescent protein (GFP) by bolus or primed infusion through the hepatic artery or portal vein. After six hours, the livers were harvested for fluorescent microscopy and histologic examination.

RESULTS—31 out of 36 liver specimens treated with IRE and the GFP plasmid demonstrated strong green fluorescence. Liver ablation by IRE was clearly demarcated on histology.

CONCLUSION—IRE is a promising technique not only for surgical tissue ablation but also for gene therapy. As IRE ablation may leave behind intact tumor antigens, these findings encourage clinical studies of tumor ablation with delivery of immunostimulatory plasmids for combined local eradication and systemic immunotherapy.

Keywords

gene therapy; hepatic malignancy; IRE; liver cancer; tumor ablation

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INTRODUCTION

Primary and secondary neoplasms of the liver are a challenge in cancer therapy. Surgical resection is the gold standard for definitive cure, yet only about 20% of patients have resectable disease. Those who undergo resection have better prognosis with a 5-year survival of 23%–38% with colorectal metastases to the liver^{1–3} and 40%–58% with hepatocellular carcinoma^{4–7}. For the majority patients who have unresectable disease or are not candidates for surgery, a variety of local ablative techniques has emerged as effective treatments, including radiofrequency ablation, cryoablation, microwave ablation, and arterial infusion. However, all patients with unresectable or recurrent disease still die of their cancer. Thus, new techniques are continuously sought to address the treatment of the visible unresectable tumors as well as microscopic residual disease, which is the culprit for recurrence.

In 1965, Coster first observed the phenomenon of a “punch-through” of the cell membrane by an electric field⁸, and the term *electroporation* was coined to describe the formation of pores in the membrane due to an electric field. In reversible electroporation, this enhanced permeability of the cell membrane is transient, and since the 1980s reversible electroporation has been employed extensively in the lab for the transfer of macromolecules generally not permeable to the target cell. For example, reversible electroporation has been used in conjunction with DNA plasmids to inhibit tumor growth in animal models. Such gene transfer allows for different antitumor strategies including apoptosis with the *TRAIL* gene, antiangiogenesis with the endostatin and angiostatin genes, and antitumor immunity with the IL-2, IL-12, IL-18, and GM-CSF genes⁹. Irreversible electroporation (IRE) was historically avoided as the upper limit of reversible electroporation as it produced permanent membrane permeability and cell death. A few years ago, perspectives changed. IRE began to be developed as a technique for ablation in the liver, pancreas, kidney, and prostate in animal models^{10–15}, and has shown success in killing hepatocellular, breast, and sarcoma tumors in animal models^{16–19}. While IRE and reversible electroporation have been studied in separate parallel paths, our interest is focused on their interaction. With distance from the center where the IRE probes are applied, electric field strength decreases incrementally. Thus, we propose that reversible electroporation exists in the periphery outside the center of IRE ablation, and that IRE can be a surgical technique that enhances gene transfer. This would hold potential as a combined treatment with ablation and a treatment targeted to the underlying disease process.

METHODS

Gene plasmid

The gene marker used to detect gene transfer was a DNA plasmid that encoded for the green fluorescent protein (GFP), which is detectable as bright fluorescent green under fluorescent microscopy. The plasmid had a beta actin promoter and an ampicillin resistance gene (gift of Dr. Dmitriy Zamarin, Memorial Sloan-Kettering Cancer Center [MSKCC]). Production of plasmid DNA was by standard transformation and purification techniques²⁰. Briefly, competent *Escherichia coli* DH5 α was transformed with the GFP plasmid by heat shock, where it was incubated in ice for 30 minutes, heated at 42°C for 90 seconds, then returned to ice for 2 minutes. The bacteria was subsequently grown at 37°C in Lysogeny Broth media, to which ampicillin was added for positive selection of the resistant transfected bacteria, and the GFP plasmid was isolated from the bacterial culture using a Qiagen plasmid isolation kit (Qiagen, Valencia, CA) and stored at –20°C. To verify the presence and function of the plasmid, Vero cells were grown in Minimal Essential Medium (MEM), transfected with the isolated GFP plasmid using lipofectamine (Invitrogen, Carlsbad, CA), incubated at 37°C, and observed for expression of GFP under fluorescent microscopy.

IRE system and parameters

IRE was delivered through the Nanoknife system (Angiodynamics, Queensbury, NY) with two monopolar 18 gauge probes spaced 1.5 cm apart, with the active tip exposed for a length of 1 cm (Figure 1). The electric parameters were 1500 V/cm, with a total of 90 pulses, a pulse length of 100 microseconds, and a pulse interval of 100 milliseconds. Nine IRE ablations were performed in the liver of each pig. A synchronization unit detected the electrocardiogram of the pig and synchronized the delivery of IRE pulses with the refractory period of the cardiac cycle to avoid arrhythmia. Pancuronium was administered intravenously to prevent muscle spasms.

Animal procedure

All animal work was approved and performed under strict guidelines by the Institutional Animal Care and Use Committee at MSKCC. Four month-old Yorkshire pigs (Animal Biotech Industries, Inc., Doylestown, PA) were obtained. For the experimental procedure, pigs were sedated with tiletamine/zolazepam (4.4mg/kg) and given buprenorphine (0.01 mg/kg) and glycopyrrolate (0.007 mg/kg). Each pig was intubated and maintained on isoflurane (1.5–3%). A midline laparotomy was performed, and wedge resections of the liver were carried out to acquire negative pre-treatment controls. The common hepatic artery was isolated through blunt dissection and cannulated with a 20-gauge angiocatheter through which 3–7 mg of GFP plasmid was administered either through a bolus injection or an infusion. Bolus injections of the plasmid were given immediately before each IRE ablative treatment. Infusions of the GFP plasmid were made of the plasmid mixed in 50 ml normal saline, and given as a loading infusion of 5 ml over 3 minutes, followed by the remaining 45 ml given at a rate of 0.75 ml/min over an hour. IRE was performed at the time points of before, 20 minutes, and 40 minutes after the plasmid infusion was started. In one pig, the portal vein instead of the common hepatic artery was cannulated, and the plasmid infusion given (Table 1).

After IRE and GFP plasmid administration, an abdominal computed tomography (CT) scan with triple phase contrast was performed. A period of 6 hours was permitted to elapse before the pig was euthanized with Euthasol (pentobarbital sodium and phenytoin sodium, 1 ml/4.5 kg) for tissue harvest of the liver. In the last pig, blood was collected before the surgery, just after the completion of the plasmid infusion and IRE, and 6 hours after treatment for laboratory tests of the liver enzymes, alkaline phosphatase, and bilirubin.

Histology and microscopy

Each liver specimen was both fixed in 10% neutral buffered formalin for histopathology as well as freshly frozen in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek, Torrance, CA) for fluorescent microscopy. Formalin fixed tissues were processed routinely, sectioned at 4 μ m thickness, and stained with hematoxylin and eosin (H&E). Frozen tissues were cut into 6- μ m sections and examined under a fluorescent microscope (Nikon Eclipse TE300, Nikon, Tokyo, Japan) equipped with a GFP emission filter to detect green fluorescence as well as a TRIT-C filter to detect autofluorescence. Images were acquired on NIS-Elements software (Melville, NY).

RESULTS

GFP fluorescence

For quality control, the isolated GFP plasmid was verified to be present and functional by the expression of strong green fluorescence under fluorescent microscopy after transfection into Vero cells. This was observed as early as 6 hours post transfection and was most robust at 24 hours.

In the pig model, 5 liver specimens were resected and acquired before any treatment, and treatment with IRE and GFP plasmid infusion was performed in 36 areas of the livers. There were no adverse events throughout the entire procedure. At 6 hours after treatment, the gallbladder was grossly fluorescent green (Figure 2A).

When examined under the GFP emission filter of the fluorescent microscope, none of the 5 pre-treatment liver specimens had any green fluorescence. On the other hand, most of the treated liver specimens demonstrated green fluorescence (Figure 2B). This green fluorescence was diffuse and scattered but was indeed present. When viewed under the TRIT-C filter of the fluorescent microscope, the areas that were fluorescent green did not show any red fluorescence, suggesting that what we saw was not autofluorescence but in fact true green fluorescence. Thirty-one of the 36 treated liver specimens had such positive green fluorescence (Figure 3A).

In a sub-group analysis of the treated specimens, positive green fluorescence was found in: all 9 specimens treated with IRE and bolus injections of plasmid; 6 out of 9 specimens treated with IRE that was performed before the plasmid infusion; 9 out of 9 specimens treated with IRE that was performed 20 minutes after the plasmid infusion was started; and 6 out of 9 specimens treated with IRE that was performed 40 minutes after the plasmid infusion was started (Figure 3B). No difference was observed between the hepatic artery and portal vein as routes of plasmid delivery.

IRE ablation

A color change from red to purple was apparent on the liver minutes after IRE ablation. H&E staining revealed that the ablated zones were well demarcated, with periportal sparing of the lobules at the periphery of the ablated zones (Figures 4A and 4B). Ablated areas are characterized by hepatocellular degeneration and necrosis, dissociation and rounding of the cells, cytoplasmic hypereosinophilia, nuclear pyknosis, and karyorrhexis (Figure 4C). This was accompanied by sinusoidal congestion and hemorrhage and neutrophilic and lymphocytic infiltration. Epithelial necrosis was observed in some small and medium blood vessels and bile ducts.

Perioperative animal well-being

None of the pigs suffered from any arrhythmia or adverse events from IRE ablation. IRE ablated lesions were depicted by low attenuation areas on non-contrast CT. On CT scan with triple phase contrast, some lesions had peripheral enhancement on the arterial phase (Figure 5). Blood tests of the last pig were unremarkable for any serious hepatitis or biliary inflammation. The ALT and AST were, respectively, 30 and 22 IU/L before surgery, 44 and 225 IU/L 1 hour after IRE ablation, and 43 and 310 IU/L 6 hours after IRE ablation, respectively. Alkaline phosphatase and total bilirubin were, respectively, 158 IU/L and 0.2 mg/dl before surgery, 167 IU/L and 0.3 mg/dl 1 hour after IRE ablation, and 167 IU/L and 0.2 mg/dl 6 hours after IRE ablation.

DISCUSSION

This study is the first of its kind to investigate the potential of gene transfer with IRE. In a 6-hour pig model, we demonstrated that IRE not only enabled ablation of the liver but also enhanced gene transfer of a locally delivered naked GFP plasmid. As verified in pre-treatment specimens, the GFP protein causing green fluorescence is not normally present in the pig liver. After treatment with IRE and GFP plasmid administration, 86% liver specimens demonstrated positive green fluorescence, and the gallbladder had turned fluorescent green. This indicates that the GFP plasmid was taken up and expressed, and the

GFP protein produced in the liver was secreted into the biliary system then reached the gallbladder. We believe the mechanism of gene transfer is that while the IRE pulses create a high electric field in the center, the electric field diminishes in strength as it radiates outward, enabling reversible electroporation and the transfer of molecules in the non-ablated periphery where cells are viable and capable of protein synthesis.

Several issues require further study. It takes a few days for cells in the IRE ablated center to complete cell death. With a time constraint of only 6 hours in this experiment, cells in the ablated center are likely still alive and may take up the GFP plasmid and express green fluorescence. A longer time course of experiment will be needed for complete necrosis and delineation of the ablated center from the live periphery where reversible electroporation and gene transfer would occur and persist. A longer time point would also allow for more robust GFP expression. While there was 100% gene transfer after treatment with bolus injections or an infusion of plasmid before IRE, the efficiency of gene transfer decreased with other treatment conditions. It is possible that optimal gene transfer requires that the plasmid is already available when IRE is performed, and that IRE is performed before the plasmid is washed away in the liver which inherently has high flow and washout. Future endeavors manipulating factors such as concentration and duration of infusion as well as time course are needed.

What is remarkable is that gene transfer and expression were achieved in as soon as 6 hours. This is an opportunistic context from an immunotherapy perspective. IRE ablated cells undergo a slow apoptotic death¹³, and at 6 hours, when the products of gene transfer are available, the cells in the ablated region will still be undergoing apoptosis with intact proteins present. If gene transfer is performed with an immune plasmid, these proteins in the ablated region may provide the antigenic stimulation for a specific immune response.

IRE ablation with gene therapy has immense potential impact for clinical application in cancer patients. Our study supports IRE to be clinically feasible, safe, and effective as a new ablation technique for immediate tissue destruction. Studies show that it can completely ablate hepatic and prostate cancer cells in vitro^{21,22} as well as induce regression of hepatocellular, breast, and sarcoma tumors in animal models¹⁶⁻¹⁹, and there are ongoing trials of IRE ablation for treatment of patients with liver, pancreatic and prostate cancer. A distinct characteristic of IRE is that it is nonthermal. Unlike radiofrequency ablation and microwave ablation, it is not subject to the heat sink effect, can ablate the tissue immediately next to large blood vessels, and may be less injurious to blood vessels and ducts¹⁰⁻¹². By altering the distance between the IRE probes, an ablation size of up to 6 cm can be reached¹⁰, making this technique useful even for large tumors in the liver. Gene transfer with IRE does not require any viral vector, making it simple and safe for the cancer patient.

We envision that combination therapy with IRE ablation and administration of an immune gene plasmid will be a synergistic approach. IRE ablation leads to immediate tumor destruction, leaving behind intact protein antigens, and the paracrine immune response brought on by the gene transfer in close proximity will be targeted towards any residual microscopic cancer. Our next step would be to study IRE in the liver with local infusion of an immune plasmid via the hepatic artery, and evaluate the uptake and expression of the gene as well as its biological immune response.

In conclusion, our study offers the first proof of principle that IRE is not only an effective ablative modality but also facilitates gene transfer. This has promising implications in cancer therapy for the destruction of tumors and the continued treatment of microscopic residual disease, and encourages study of IRE with immune plasmids for targeted local and systemic immunotherapy.

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Figure 1.
IRE ablation of the liver.

Figure 2. Gross appearance of the gallbladder (A) and fluorescent microscopy of the liver (B)
The gallbladder turned a fluorescent green after IRE and GFP plasmid administration. This treatment also resulted in expression of green fluorescence in the liver; image was taken from a representative frozen section under fluorescent microscopy at 100 x magnification.

Figure 3. Prevalence of GFP fluorescence overall (A) and by group (B)

There was no GFP fluorescence in negative controls and 86% GFP fluorescence in specimens treated with IRE and GFP plasmid administration. GFP fluorescence was evident in 67% of specimens with IRE before GFP plasmid infusion, 100% of specimens with IRE immediately after a GFP plasmid bolus, 100% of specimens with IRE after 20 minutes of GFP plasmid infusion, and 78% of specimens with IRE after 40 minutes of GFP plasmid infusion.

Figure 4. H&E stain of IRE ablations in the liver

IRE ablation zones are demarcated from unablated areas (A, bar represents 1 mm), with some periportal sparing of the hepatic lobule at the periphery (B, bar represents 100 μm). Ablated areas have massive hepatocellular degeneration and hemorrhage (C, bar represents 20 μm).

Figure 5. CT scan of the liver after IRE ablation
IRE ablated lesions in the liver demonstrated peripheral enhancement on the arterial phase of a triple contrast CT.

Table 1

Experimental procedure

Route	Manner of plasmid administration	Timing of IRE
Hepatic artery	Bolus	Immediately after bolus
Hepatic artery	Infusion	Before, 20 minutes after, and 40 minutes after the infusion is started
Portal vein	Infusion	Before, 20 minutes after, and 40 minutes after the infusion is started