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Electrically mediated plasmid DNA delivery to hepatocellular carcinomas in vivo

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Gene therapy by direct delivery of plasmid DNA has several advantages over viral gene transfer, but plasmid delivery is less efficient. In vivo electroporation has been used to enhance delivery of chemotherapeutic agents to tumors in both animal and human studies. Recently, this delivery technique has been extended to large molecules such as plasmid DNA. Here, the successful delivery of plasmids encoding reporter genes to rat hepatocellular carcinomas by in vivo electroporation is demonstrated. Gene Therapy (2000) **7,** 826–829.

Keywords: *electroporation; in vivo; gene delivery; rat hepatocellular carcinoma*

Safe and efficient methods for *in vivo* DNA delivery at diverse expression levels and durations are needed for the effective treatment of a variety of diseases. Current *in vivo* delivery methods can be broadly divided into viral and nonviral categories. Although viral vectors induce long-term, high gene expression, limitations such as the possibility of insertional mutagenesis or induction of the host immune response limit the usefulness of viral delivery.[1]

Mouse skeletal muscle injected with plasmid DNA alone results in long-term protein expression.[2] Plasmid DNA is neither replicated nor integrated into the host cell genome, but remains in its episomal form[3] and is expressed in both dividing and nondividing cells. The injection of DNA does not result in the production of anti-DNA antibodies,[4,5] which allows for multiple treatments. The resulting expression may be short term compared with viral delivery, which may be an advantage if long-term expression is not desirable.

In all *in vivo* gene therapy techniques, reproducible delivery and control of molecule dosage are common problems. Since the efficiency of gene transfer by naked DNA injection is lower than that of viral delivery, both chemical and physical techniques have been used to increase the efficiency of DNA uptake and expression. *In vivo* liposome-mediated delivery of plasmid DNA was first described in 1983[6] and is used in many clinical trials. In particle bombardment or 'gene gun' transfer, the target tissue is bombarded with DNA coated gold particles, which penetrate the cells.[7]

Electroporation is a recently developed method using electric pulses to enhance cellular uptake of extracellular molecules. This technique has been used to deliver drugs successfully to treat several types of tumors in both animal and human studies.[8,9] Electrically enhanced *in vivo* plasmid gene delivery to mouse skin cells was first demonstrated in 1991[10] and is more effective than liposome delivery or particle bombardment.[11] This method has recently been used to deliver reporter genes *in vivo* to normal rat hepatocytes,[12,13] rat brain tumor cells,[14] mouse testes,[15] mouse melanoma cells,[16] and skeletal muscle.[17–19]

A previous study by this laboratory demonstrated *in vivo* gene delivery to normal liver tissue using electroporation.[12] In the present study, the delivery of plasmids encoding reporter genes to a solid visceral tumor is demonstrated. Reporter genes were used to evaluate delivery of plasmid DNA to rat hepatocellular carcinomas. Since these fast growing tumors reach a mean size of 345 mg by 48 h after treatment, this time-point was used for these experiments. In order initially to demonstrate the enhancement of DNA delivery to these tumors by electroporation, conditions similar to those previously described for delivery of bleomycin to hepatocellular carcinomas with electroporation were used.[8] This same pulsing protocol enhanced luciferase expression nine-fold over plasmid DNA injection alone in normal liver tissue.[12] In rat hepatocellular carcinomas, the application of a similar pulsing protocol resulted in a 15-fold increase in luciferase activity over simple injection (Figure 1). Thus for rat hepatocellular carcinomas, the same pulsing protocol used to deliver chemotherapeutic agents effectively enhanced plasmid DNA delivery *in vivo*.

Electroporation is a threshold phenomenon. The field strength necessary for molecule delivery must exceed a threshold value. Moderate increases in the applied field strength result in delivery; however, larger increases in the applied field from this threshold result in tissue damage. With this constraint in mind, the effect of changes in pulse field strength on gene delivery were explored (Figure 2). These experiments revealed that pulses of 750 V/cm did not significantly increase luciferase activity. However, 1000 and 1250 V/cm pulses induced

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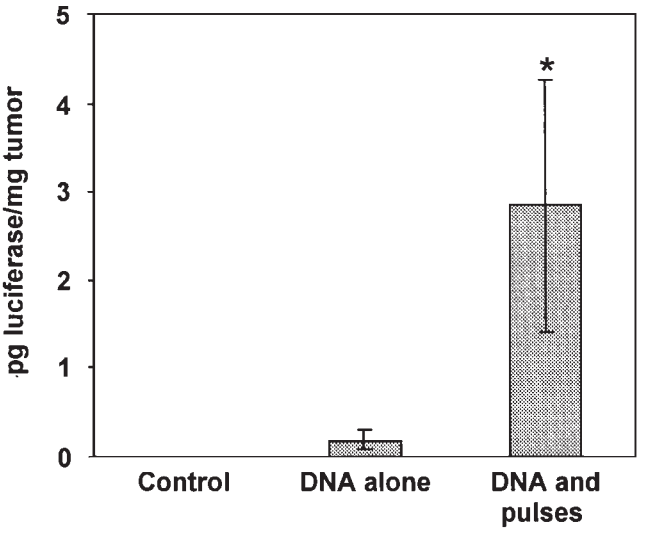

Figure 1 *Enhancement of plasmid DNA delivery to rat hepatocellular carcinomas as determined by luciferase expression. Male Sprague–Dawley rats were placed in an induction chamber charged with 5% isoflurane in O_2. After anesthetization, rats were fitted with a standard rodent mask and kept under general anesthesia with 3% isoflurane. The liver was surgically exposed and injected with 10^6 N1S1 rat hepatoma cells (ATCC CRL1604) in 50 μl PBS. Tumors were allowed to grow for 7 days. Following tumor growth, the animals were anesthetized as above and the liver with tumors surgically exposed. Tumors were measured using a digital caliper and tumor volume calculated by the formula $v = abc\pi/6$, where a, b, and c are the three mutually orthogonal diameters, yielding an average tumor volume of 80 mm^3. pCMV-Luc+[14] was prepared using Qiagen Megaprep kits (Qiagen, Valencia, CA, USA), then diluted in sterile injectable saline. Tumors were injected with 1 μg plasmid per mm^3 tumor volume in an injection volume equal to one half the tumor volume. Electric pulses were applied at a field strength of 1250 V/cm immediately after injection using a custom designed applicator containing seven electrodes, placed in three parallel rows, spaced to allow strategic placement of the electrodes around and into the tumor and a CytoPulse PA4000 DC Generator (CytoPulse Sciences). A total of 14 pulses (100 μs, 1 Hz) were applied in a seven-step sequence through the different electrodes. At each sequence step, the field was moved around the tumor by activating 2, 3, or 4 of the seven electrodes. Previous work had demonstrated that rotating a field was more effective.[22] After pulses, the animals were immediately closed with surgical staples. Animals were humanely killed, 48 h after plasmid delivery and the tumors were excised. For luciferase quantitation, the tumors were weighed and then homogenized in buffer (50 mM K_3PO_4, 1 mM EDTA, 1 mM DTT, 10% glycerol) using a Tissumizer (Tekmar, Cincinnati, OH, USA). Extracts were assayed for luciferase activity[23] and quantitated using a MLX microtiter plate luminometer (Dynex Technologies, Chantilly, VA, USA). Activity was normalized to the tumor weight and expressed relative to injection alone. Each bar represents the mean of three independent experiments with three tumors treated per experiment, for a total of nine tumors. Error bars are the standard error of the mean. Statistical significance ($P \leq 0.05$) relative to injection alone is noted by an asterisk.*

a 14- to 16-fold increase in plasmid DNA expression over injection alone. Peak expression levels (30-fold) were observed when 1500 V/cm pulses were applied. At a higher field strength, 2000 V/cm, higher luciferase activity was observed, but visible tissue damage was noted in the normal liver tissue in the form of a highly localized avascular region immediately adjacent to the electrode insertion points. No significant damage to normal tissue in response to 100 μs electric pulses is noted at 1500 V/cm or less.[20] Since high levels of expression were observed at 1500 V/cm and no damage was noted, ensuing experiments were performed at this voltage.

The *in vivo* electroporation experiments described thus

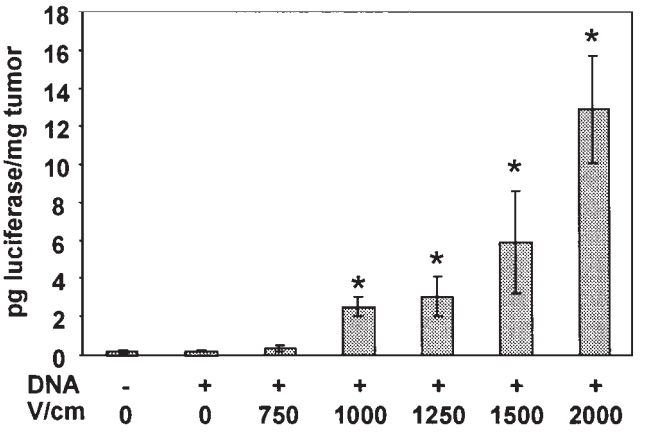

Figure 2 *Effect of pulse field strength after administration of plasmid DNA. Tumors were induced, injected with plasmid DNA followed by pulses of varying field strength and analyzed for luciferase expression as in Figure 1. Bars represent the mean and standard error of the mean from four independent experiments, each containing three tumors per treatment group, for a total of 12 tumors. Statistical significance ($P \leq 0.05$) relative to injection alone is noted by an asterisk.*

far were performed after the injection of 1 μg DNA per mm^3 tumor volume. This amount of DNA resulted in easily quantifiable luciferase expression. DNA dose dependence was investigated in order to maximize tumor reporter protein expression. Quantities ranging from 0.1 to 2 μg per mm^3 tumor volume were examined (Figure 3). Luciferase expression showed a DNA dose dependence, with the highest tested activity at 2 μg per mm^3 tumor volume.

To determine the cellular distribution of delivery within the tumor, histochemical staining for β-galactosidase was performed (Figure 4a and b). Tumors treated with both plasmid and electric fields were positive for expression as indicated by the blue stained cells.

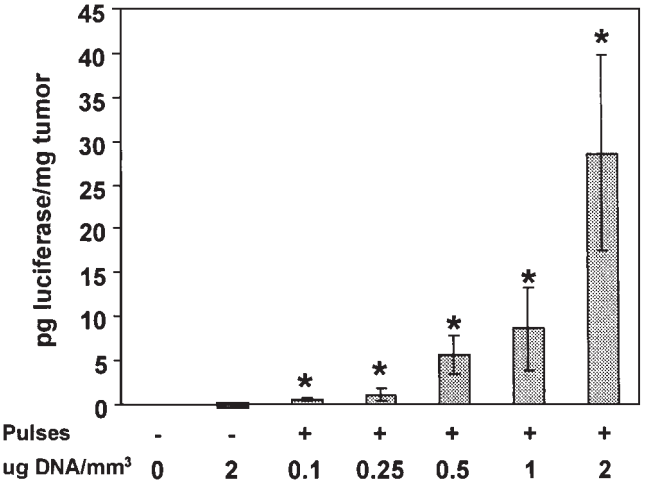

Figure 3 *Dependence of luciferase expression on amount of plasmid DNA injected. Tumors were injected with varying concentrations of plasmid DNA in the same volume of saline normalized to tumor volume, followed by pulses at 1500 V/cm. Luciferase activity was determined as described in Figure 1. Bars represent the mean and standard error of the mean from three independent experiments, each containing three tumors per treatment group, for a total of nine tumors. Statistical significance ($P \leq 0.05$) relative to injection alone is noted by an asterisk.*

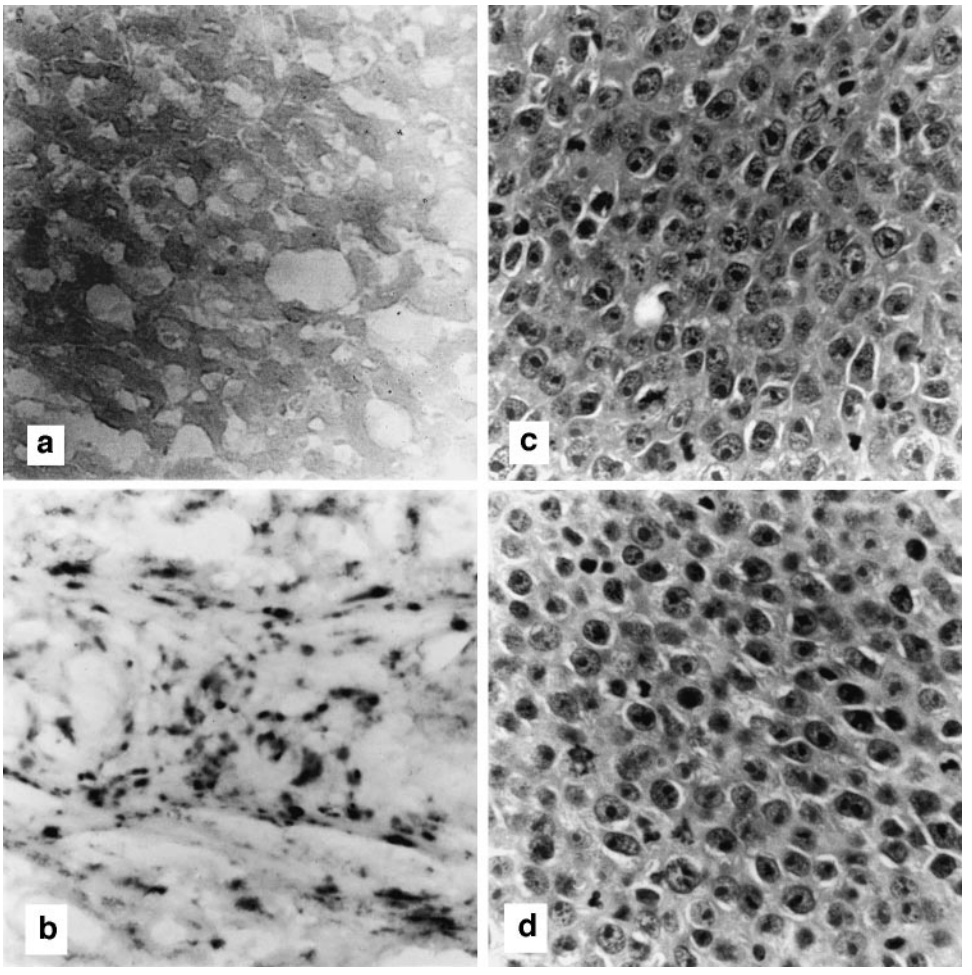

Figure 4 *(a, b) Histochemical staining for β-galactosidase expression in frozen sections. Tumors were induced in rats and injected with pSV-βGal (Promega, Madison, WI, USA). After 48 h, animals were humanely killed and the tumors were excised and fixed in 0.5% glutaraldehyde. β-Galactosidase activity was detected in 15 μm-thick cryosections by staining in 15 mM $K_4Fe(CN)_6$, 15 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, and 1 mg/ml X-gal in PBS for 12 h. (a) pSV-βGal injection only; (b) injection of pSV-βGal followed immediately by pulses at 1500 V/cm as described previously; (c,d) histologic analysis of paraffin-embedded sections by hematoxylin and eosin (H&E) staining after 48 h from (c) a tumor that did not receive pulses, and (d) a tumor which received the same electrical treatment as in (b). Unpulsed and pulsed tumors both revealed sheets of viable cells with increased nuclear to cytoplasmic ratio, pleomorphic nuclei with prominent eosinophilic nucleoli, and numerous mitotic figures. Rare single apoptotic cells were identified, and there was no necrosis present.*

Expression was noted only in the area that received electroporation. No expression was noted in tumors that received plasmid injection only or no treatment. Examination of hematoxylin and eosin stained sections from tumors that were unpulsed and pulsed (Figure 4c and d) revealed viable tumor cells with no evidence of necrosis and minimal single cell apoptosis for both types of samples. This confirmed that electrical pulses alone caused no significant damage to the tumor tissue.

These data corroborate previous studies that short length (μs) pulses efficiently enhance delivery of DNA to tissues such as normal hepatocytes[12] and rat brain tumor cells.[14] Longer length (ms) pulses also result in increased expression of injected plasmids to mouse testes,[15] rat hepatocytes,[13] mouse melanoma cells[16] and mouse skeletal muscle,[17–19] but tissue damage is observed at field strengths greater than 100 V/cm.[13,15]

The results presented here demonstrate that *in vivo* electroporation enhances delivery of plasmid DNA to rat hepatocellular carcinomas. This delivery technique could eventually be adapted for clinical studies by performing the molecular delivery using laparotomy. Detectable levels of non-reporter cDNA expression have been reached after augmentation of intratumor plasmid DNA delivery by *in vivo* electroporation. Transfer of plasmid DNA by electroporation of a plasmid encoding monocyte chemoattractant protein 1 into rat brain tumors resulted in increased numbers of tumor monocytes.[14] Electroporation of a plasmid encoding a Stat3 dominant negative variant into pre-existing mouse melanomas inhibited tumor growth and induced tumor regression.[21] Therapeutic levels have also been reached after electroporation of healthy tissue. Erythropoietin cDNA expression after muscle injection was increased to levels adequate to induce an increase in hematocrit in mice.[19] An increase in IL-5 protein expression was observed after muscle electroporation.[17] It is apparent that protein expression observed after *in vivo* electroporation is ample to induce quantifiable responses *in vivo*. One major benefit of this technique is that protein expression is focused on the area electroporated. In tissues other than muscle, plasmid delivery, even enhanced, may turn out to result in only

short-term expression. This may be useful in cases where long-term expression of a molecule such as an immune inducer would be toxic. Electroporation offers a highly reproducible method of *in vivo* plasmid delivery which is a complement to liposome and gene gun delivery and an alternative to viral delivery.

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