Gene Ther Mol Biol Vol 5, 55-60, 2000

Delivery of plasmid DNA by *in vivo* electroporation

Review Article

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Key words: gene therapy, electroporation

Received: 13 June 2000; accepted: 13 July 2000; electronically published: February 2004

Summary

For gene therapy, *in vivo* delivery of plasmid DNA offers an alternative to viral delivery methods. Since the efficiency of plasmid delivery to tissues is generally lower than viral delivery, several methods have been introduced to augment *in vivo* plasmid delivery including liposome conjugation, particle mediated delivery, and electroporation. *In vivo* electroporation has reached clinical trials for the delivery of chemotherapeutic agents to cancers, and a number of preclinical studies have been performed demonstrating that this technique also enhances plasmid delivery and expression of both reporter and therapeutic genes or cDNAs. This delivery has been performed to a number of tissues including skin, muscle, liver, testes and tumors employing a wide range of electrical conditions and electrodes. While this preclinical research is promising, further optimization of electrical conditions and electrodes may be necessary for clinical use. With the availability of a variety of therapeutic gene delivery techniques, it will be possible to tailor gene therapies to individual diseases.

I. Introduction

Gene therapy has the potential to play a role in the effective treatment of a variety of diseases. Biological gene therapy employs genetically engineered viruses to deliver the desired gene or cDNA. Several types of viral vectors including recombinant retroviruses, adenoviruses, and adeno-associated viruses have been described or employed in gene delivery (Kay, 1997). While gene therapy with these vectors may result in high protein levels and long term expression, short term, lower levels of expression of molecules such as immune modifiers may also be desirable. Ideally, multiple gene delivery techniques may be necessary to fit multiple treatment regimens.

The delivery of plasmid DNA encoding the gene or cDNA of interest may also be used for gene therapy. Plasmid DNA is neither replicated nor integrated into the host cell genome, but remains in its episomal form (Nichols et al, 1995), and expression is generally short term in tissues other than muscle. DNA injection does not result in the production of anti-DNA antibodies (Jiao et al, 1992; Robertson, 1994), which allows for multiple treatments. Since the efficiency of plasmid delivery is lower than that of viral delivery, several methods have been introduced to increase delivery *in vivo*, including liposomes, microparticle bombardment and electroporation.

During *in vivo* electroporation, electric pulses are applied directly to the tissue to enhance uptake of extracellular molecules (Jaroszeski et al, 2000). The delivery of chemotherapeutic agents to tumors by this method was first demonstrated in subcutaneously injected hepatocellular carcinomas (Okino et al, 1987). This technique, termed electrochemotherapy, results in substantial, but localized tumor necrosis. Electrochemotherapy is a highly effective anti-tumor regimen and has been demonstrated preclinically in variety of cutaneous and internal tumors, including rat (Jaroszeski et al, 1997a) and rabbit (Ramirez et al, 1998) hepatocellular carcinomas, Lewis lung carcinoma (Kanesada, 1990), sarcomas and melanomas (Mir et al, 1991), mammary tumors (Belehradek et al, 1991), gliomas (Salford et al, 1993), fibrosarcomas (Sersa et al, 1995), and melanomas (Heller et al, 1995). This work was extended to clinical trials in head and neck squamous cell carcinoma, melanoma, basal cell carcinoma, Kaposi's sarcoma, and adenocarcinoma (Heller et al, 1999; Jaroszeski et al, 1997b). In these clinical trials, objective responses varied from 72 to 100%.

Table 1. Luciferase expression after muscle electroporation of plasmid DNA

	Mir et al, 1999	**Mathiesen et al, 1999**	**Vicat et al, 2000**	**Lucas et al, submitted**
Pulse description	Square LVLP	Bipolar trains	Square HVSP	EEP
Electrode	Caliper	Wire pair	Caliper	Needle
µg plasmid	15	25	10	100
Muscle treated	mouse tibial cranial (transcutaneous)	rat soleus (surgically exposed)	mouse tibialis anterior (exposed)	mouse gastrocnemius (transcutaneous)
Fold increase in expression	100	16	500	1000
Peak measured expression	Day 7	Day 3	Day 1	Day 2
Observed length of expression	9 months	3 days	6 months	7 days

LVLP, low voltage long pulses; HVSP, high voltage short pulses; EEP, exponentially enhanced pulses

II. Reporter gene delivery with *in vivo* electroporation

Delivery of DNA to cells by the application of electric pulses was first reported in 1982 (Wong and Neumann, 1982). *In vivo* electroporation to enhance plasmid delivery was later demonstrated in the skin cells of newborn mice using exponential pulses with a clip electrode (Titomirov et al, 1991). Optimal transformation of skin cells was noted after two pulses in opposing polarities of a field strength of 300-400 V/cm and a pulse length of 0.1-0.3 ms. In experiments using three much longer, higher amplitude exponential pulses and the addition of pressure with a caliper electrode (1200V/cm, 10-20 ms), β-galactosidase expression was detected to a depth of 370 µm in the skin of hairless mice after topical administration of plasmid DNA (Zhang et al, 1996). More recently, delivery to skin with square wave pulses after intradermal injection has been demonstrated (Heller et al, submitted). While plasmid delivery with caliper electrodes and eight short (0.1 ms), high field strength (1500 V/cm) pulses increased luciferase reporter expression10 fold in the treated skin 24 hours after treatment, no increase was seen using eight long (20 ms) lower field strength (100 V/cm) pulses. Skin delivery of a plasmid encoding IL12 induced a systemic response as well, in the form of increased serum levels of interferon γ. In these experiments, plasmid delivery with a custom designed electrode induced higher expression than delivery with simple calipers. These results emphasize the importance of optimizing both pulsing protocols and electrode configurations with respect to tissue type to avoid damage and maximize protein expression.

Electroporation enhances plasmid expression in other tissues. Delivery to normal rat liver was first characterized using six 0.1 ms pulses at a variety of field strengths using a 6 needle array (Heller et al, 1996). Maximum luciferase expression occurred at 1000 to 1500 V/cm, electrical conditions very similar to those used for drug delivery in clinical trials. 48 hours after treatment, approximately 30% of cells in the electroporated area expressed β-galactosidase and this expression was still detectable 21 days later. The expression was dose dependent, with peak expression occurring with delivery of 25 µg plasmid. GFP expression in normal liver from delivery of plasmid DNA was examined using pulses that were less intense but 500 fold longer (eight 50 ms at a variety of field strengths) and a disk electrode (Suzuki et al, 1998). The highest expression was noted at 250 V/cm, while the extreme damage noted at 500 V/cm probably contributed to the lack of expression. This expression was 3.5 fold higher than with 50 ms pulses than with 25 or 99 ms pulses at this field strength. These investigators found a DNA dose dependent expression to 80 µg plasmid DNA, which may be due to the different electroporation parameters used. Clearly, different pulsing parameters may be used to effectively enhance plasmid DNA deliver to the same tissue.

Delivery by lipid conjugation, microparticle bombardment and electroporation using equal quantities of plasmid DNA was directly compared in chicken embryos (Muramatsu et al, 1997a). For liposome-mediated delivery, DNA was complexed per manufacturer's instructions (Lipofectamine, Gibco BRL, USA) and injected. In microparticle bombardment, DNA was bound to 0.9 mg tungsten beads and delivered by nitrogen gas at 20 kfg/cm^2 at a distance of 6 cm. For electroporation delivery, tissue received three 50 ms pulses at 31.25 V/cm with a plate electrode after plasmid injection. No significant difference was noted in embryo viability or number of embryos positive for β-galactosidase staining 48 hours after delivery, although electroporation appeared to transfect a larger area with a greater intensity of expression.

Using CAT as a reporter, this group also compared several electroporation conditions for optimization of DNA delivery in surgically exposed mouse testes (Muramatsu et al, 1997b). In each case, eight low voltage, long pulses were delivered. Tissue damage due to heat generation was observed after severe conditions such as 100V for 50 ms, so only conditions that caused minimal damage were presented.

The optimal conditions elucidated in this experiment were 50 V for 10ms or 25 V for 50 ms. Furthermore,

Table 2. Serum protein expression after muscle electroporation of plasmid DNA

	Aihara et al, 1998	**Rizzuto et al, 1999**	**Maruyama et al, 2000**	**Widera et al, 2000**
Pulse description	Square LVLP	Bipolar trains	Square LVLP	Square LVLP
Electrode	Needle pair	Parallel wires	Needle pair	Needle pair
Muscle treated	Mouse tibialis anterior (transcutaneous)	Mouse quadriceps (exposed)	Rat thigh (exposed)	Mouse tibialis anterior (transcutaneous)
Fold increase in expression	119	>100	7	400
Peak measured expression	Day 5-7	Day 7	Day 7	Day 5
Observed length of expression	< 42 days	84 days	>21 days	20 days
Reporter	Interleukin-5	Erythropoietin	Erythropoietin	HBsAg

LVLP, low voltage long pulses; mice received 50 μg plasmid; rats received 400 μg plasmid

CAT activity was examined after liposome, microparticle bombardment, and electroporation deliveries to mouse testes (Muramatsu et al, 1998). Microparticle bombardment and electroporation both significantly increased expression, while liposome delivery had no significant effect on expression.

Much of the focus of *in vivo* electroporation has been on muscle delivery. Mouse skeletal muscle injected with plasmid DNA alone expressed reporter genes in both dividing and non-dividing cells (Wolff et al, 1990). This expression can be significantly augmented by the addition of electric pulses (Aihara et al, 1998). In addition, individual variability in transgene expression is reduced after electroporation (Mir et al, 1999). The data from several groups that have examined luciferase reporter expression after muscle electroporation is summarized in **Table 1**. Mir et al, (1999) found maximum luciferase expression after delivery of eight 20 ms pulses at 200 V/cm with a caliper electrode in mouse tibial cranial muscles. This expression lasted 9 months. Electroporation also enhanced luciferase plasmid DNA expression in various mouse, rat, and rabbit muscles, and expression of FGF1 was detected in muscle after plasmid electroporation of mouse and monkey muscle. Similar long, low voltage pulses (ten 40 ms) were compared to trains of bipolar pulses (ten 1000 Hz trains of on thousand 0.4 ms pulses each), both at a field strength of 100-180 V/cm (Mathiesen, 1999). The pulsing protocols, which resulted in the same total pulse time, induced similar levels of expression. Although more muscle damage was observed after the long pulses, the muscle cells had regenerated by 2 weeks after treatment.

While one group found a 50 fold decrease in luciferase expression after plasmid delivery with eight 0.1 ms 1200 V/cm pulses (Mir et al, 1999), another found increasing expression with increasing field strength with 0.1 ms pulses to an expression maximum at 1800 V/cm (Vicat et al, 2000). This expression increased linearly with DNA concentration up to 50 μg. This group also reported more stable expression (>6 months) with shorter pulses. This difference may be due to the more intense immune infiltrate observed after longer pulses. A 20 fold increase in expression after short, high voltage electroporation delivery was confirmed using 0.1 ms pulses at 1500 V/cm (Lucas et al, submitted), although in these experiments, long, low voltage pulses or exponentially enhanced pulses induced an even larger (1000 fold) increase in luciferase expression. Since these groups compared similar pulsing parameters, other variables such as the electrode used may be affecting the level of delivery. Clearly, though, plasmid delivery to muscle is enhanced by several different pulse types.

In vivo muscle electroporation also enhances systemic expression of plasmid DNA. A comparison of electroporation enhanced serum protein expression after *in vivo* electroporation is summarized in **Table 2**. Since several different serum reporter plasmids were used, the increase in expression noted may not be directly comparable. Electroporation significantly increased serum expression of interleukin-5 after muscle injection (Aihara et al, 1998). In this case, three 50 ms 100 V/cm square wave pulses were delivered with a needle pair. Serum expression of mouse erythropoietin was demonstrated after electroporation of as little as 1 μg plasmid DNA into muscle with ten 1 second pulse trains of on thousand square bipolar pulses (0.2 ms each, 90 V/cm, Rizzuto et al, 1999). Erythropoietin levels peaked at day 7 and were elevated for at least 84 days. No muscle damage from electroporation was detected histologically 24 hours after treatment. At one week, areas of muscle fibers with central nuclei and small necrotic areas with lympocyte infiltrations were observed, constituting up to 10-20% of the total electroporated muscle. At one month after treatment, muscles appeared normal. A plasmid encoding rat erythropoietin was also delivered to rat muscle at 4 sites with 100 μg plasmid each with eight 50 ms 200 V/cm pulses delivered with a needle pair (Maruyama et al, 2000). In this case, serum erythropoietin expression

peaked at day 7 and continued for 32 days, with a corresponding increase in hematocrit continuing at least to day 32. Systemic expression of a hepatitis B surface antigen was also observed after *in vivo* electroporation of 50 μg plasmid (Widera et al, 2000). After delivery of six 50 ms 100 V/cm pulses with a needle pair, antigen expression peaked 5 days after treatment and continued at least 20 days post injection in nude mice, while this expression began to wane at day 13 in immunocompetent mice, possibly due to a cellular immune response against transfected muscle cells or clearance of antigen in antigen-antibody complexes. Electrically enhanced gene transfer to muscle, which can result in both local and systemic transgene expression, may prove an effective tool for treating a variety of diseases.

A wide range of electrical conditions has also been used to enhance plasmid delivery directly to several tumor types. Increased plasmid encoded β-galactosidase expression was first demonstrated after intra-arterial injection of plasmid DNA, followed by eight 0.099 ms 600 V/cm electric pulses applied directly to rat brain tumors (Nishi et al, 1996). Plasmid delivery by hemagglutinating virus of Japan (HSV) liposomes, microparticle bombardment and electroporation was compared in rat bladder cancers (Harimoto et al, 1998). In this experiment, eight 50 ms pulses at a field strength of 143-1000 V/cm were applied with a needle type electrode. These researchers found that all three delivery methods may be suitable for therapy of localized bladder tumors. Expression of β-galactosidase was also detected in B16 mouse melanomas after intratumor injection of 12 μg plasmid and application of ten 5 ms pulses at 800-900 V/cm with caliper electrodes (Rols et al, 1999). No gene transfer was detected in these experiments with short (μs) pulses. In contrast, 15% of B16 melanoma cells were positive for β-galactosidase expression after intratumor delivery of 100 μg plasmid with fourteen 0.1 ms pulses at a field strength of 1500 V/cm (Niu et al, 1999). In these experiments, pulses were delivered segmentally so that each tissue area received only two pulses. The significant difference between these two results may be due to different electrode configurations or to the use of different reporter plasmids. Using 0.1 ms pulses, a plasmid encoding luciferase was delivered to rat liver tumors (Heller et al, 2000). Expression at 48 hours was highest at 2000 V/cm, although this extreme field strength burned the tissue immediately around the electrodes. Therefore, 1500 V/cm, which caused no evidence of tissue damage, was used for subsequent experiments. The level of luciferase expression in response to electroporation increased as the amount of plasmid increased up to 2 $\mu g/mm^3$ initial tumor volume. Histochemical staining for β-galactosidase expression was also enhanced by electroporation **(Figure 1)**. In an interesting set of experiments in mouse breast tumors, *in vivo* electroporation of lipid complexed and naked DNA was compared (Wells et al, 2000). Using caliper electrodes and six 1 ms pulses, luciferase expression was augmented 2 orders of magnitude maximally 48 hours after delivery at 1100 V/cm. No statistical difference in expression was noted between electroporation of naked plasmid or lipoplexes.

III. Preclinical gene therapy with *in vivo* electroporation

Electroporation enhanced plasmid delivery to muscle may induce a clinically relevant response. After delivery of a plasmid encoding erythropoietin, the responding hematocrit is significantly increased in mice up to 6 months (Rizzuto et al, 1999) after delivery of as little as 1 μg plasmid or in rats up to 32 days after delivery of 400 μg plasmid (Maruyama et al, 2000). An immune response to viral proteins can also be induced after muscle electroporation of plasmid DNA. In mice, strong antibody responses to HbsAg and to HIV gag protein were detected two weeks after delivery of 3 μg or 10 μg plasmid respectively (Widera et al, 2000). In addition, four weeks after plasmid delivery, an anti-gag T cell response was observed after challenge with a vaccinia virus expressing HIV gag. Although there is no consensus as to the "best" muscle electroporation conditions, the experiments described here demonstrate that electroporation of plasmid DNA into muscle has potential as a gene therapy in a clinical setting, enhancing both intramuscular and systemic transgene expression.

Electroporation enhanced plasmid delivery directly to tumors may also induce a clinically relevant response. After delivery of a plasmid encoding human monocyte chemoattractant protein-1 into rat brain tumors, large numbers of macrophages and lymphocytes were observed in the tumor tissues (Nishi et al, 1996). Electroporation enhanced delivery of a plasmid encoding a dominant negative Stat3 variant into B16 mouse melanomas inhibited tumor growth and caused tumor regression mediated by apoptosis (Niu et al, 1999). The combination of electrochemotherapy and cytokine plasmid delivery by electroporation into B16 melanomas prevents tumor recurrence and induces long term antitumor immunity in mice (Heller et al, submitted). After electroporation of plasmids encoding diphtheria toxin or herpes simplex thymidine kinase followed by gancyclovir administration, the growth of subcutaneously inoculated colon adenocarcinomas was significantly inhibited (Goto et al, 2000).

IV. Conclusions and clinical considerations

While electroporation is highly effective at enhancing tissue expression of transgenes, it will be necessary to optimize two variables for plasmid DNA delivery, quite possibly for each tissue or tumor type: the pulsing conditions and the electrodes used for pulse delivery.

Several pulse shapes will enhance DNA delivery *in vivo*, including exponential, square wave, and bipolar trains. In each case, the total number, length, and field strength of each pulse can be varied considerably. Effective pulse lengths range from 0.1 to 50 ms, while effective field strengths range from 200 to 1800 V/cm.

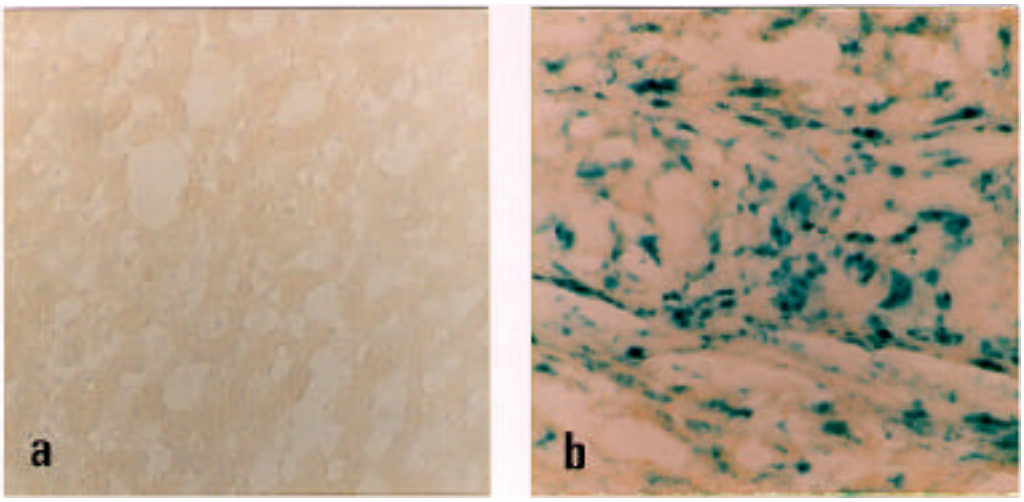

Figure 1. Histochemical staining for β-galactosidase expression in rat hepatocellular carcinomas (Heller et al, 2000). 15 μm frozen sections were stained for expression 48 hours after delivery. (**a**) Injection only of 50 μg pSV-βgal (Promega, Madison, WI, USA); (**b**) Injection of 50 μg pSV-βgal followed by two 100 μs pulses at 1250 V/cm per tissue area.

The potential for damage resulting from delivery, which may result from irreversible electroporation or from heat damage after long or high intensity pulses, must be balanced against the increased DNA expression. This damage may be much more important in plasmid delivery to healthy tissues such as muscle than delivery directly to tumor tissue.

The potential damage from electric pulses to rabbit liver, pancreas, kidney or spleen after delivery of eight 0.1 ms pulses at a field strength of 850 V/cm was assessed histologically (Ramirez et al, 1998). Immediately after pulse application, the primary effect was edema formation. At days 2 and 7 after pulse delivery, localized necrosis and fibrosis was limited to the electrode contact sites. In depth studies of the effect of six 0.1 ms 1300V/cm pulses on skin, muscle, nerves, and blood vessels in the hind limbs of rats were also performed (Richard Heller, personal communication). Histological analysis of the tissues after one to three treatments showed short term (3 days), localized necrosis to skin and muscle that started to resolve within 14 days and completely resolved by 56 days after treatment. In addition, all animals regained full limb function within 6 minutes after the therapy was administered. For clinical consideration, detailed experiments elucidating damage from long, low voltage pulses will also be necessary.

Electrode design must also be optimized (Gilbert et al, 1997). Caliper or plate electrodes are easily available and simple to use, although the treated tissue must be accessible and in each case the voltage must be calculated based on the thickness of tissue "gripped". In this case, electric pulses may be delivered over a large surface area, but this delivery also requires higher voltages to maintain the field strength. Needle electrodes increase the depth of delivery and allow treatment of tissue without altering tissue shape. The treated area can be better defined as well, and the specific voltage used can be standardized from sample to sample. Particularly for clinical use, it will be necessary to design pulsing protocols and electrodes specifically for each application of gene therapy.

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