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L. Heller
Old Dominion University, lheller@odu.edu

D. Coppola

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BRIEF COMMUNICATION

Electrically mediated delivery of vector plasmid DNA elicits an antitumor effect

L Heller[1] and D Coppola[2]
[1]*Center for Molecular Delivery, University of South Florida, Tampa, FL, USA; and* [2]*Department of Pathology, University of South Florida College of Medicine, Tampa, FL, USA*

In vivo electroporation is an efficient means of increasing plasmid DNA delivery to normal tissues, such as skin and muscle, as well as directly to tumors. In the experiments described here, plasmid DNA was delivered by in vivo electroporation to B16 mouse melanomas using two very different pulsing protocols. Reporter expression increased 21- or 42-fold, respectively with electroporation over injection alone. The growth of experimental melanomas with an approximate diameter of 4 mm on the day of treatment was monitored after electroporation delivery of reporter plasmid DNA. Remarkably, short-term complete regressions using one of these pulsing protocols occurred in up to 100% of mice. These regressions continued long term in up to 83% of animals. 70% of these mice were resistant to challenge with B16 melanoma cells. Histological analysis revealed large numbers of apoptotic cells 24 h after treatment. This antitumor effect did not require therapeutic cDNA expression or eukaryotic sequences.
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Keywords: *in vivo electroporation; gene therapy; plasmid DNA; experimental melanoma*

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 *in vivo* electroporation, which has been used in phase II clinical trials for the delivery of chemotherapeutic agents to cancers.[1,2] 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 to many tissues.[3]

Electroporation delivery has been performed to a number of normal tissues including skin,[4–7] liver,[8,9] and testes,[10] although since skeletal muscle injected with plasmid DNA alone expresses reporter genes,[11] much of the focus of *in vivo* electroporation has been on muscle delivery. Both intramuscular and systemic plasmid expression are significantly augmented by the addition of electric pulses.[12–23]

Electroporation directly into tumors also enhances plasmid expression. This has been demonstrated using reporter genes in rat brain tumors,[24] mouse melanomas,[25] mouse mammary tumors,[26] and rat liver tumors.[27] Therapeutic responses have also been described, including human monocyte chemoattractant protein-1 in rat brain tumors,[24] a dominant negative Stat3 variant in mouse melanomas,[28] diphtheria toxin or herpes simplex thymidine kinase in subcutaneously inoculated colon adenocarcinomas,[29] and IL12 in subcutaneously implanted hepatocelluar carcinomas,[30] subcutaneously implanted colon carcinomas,[31] or IL12 and IL18 in mouse melanomas.[32] The combination of electrochemotherapy and cytokine plasmid delivery by electroporation into mouse melanomas prevents tumor recurrence and induces long-term antitumor immunity.[33] Intramuscular delivery of plasmids encoding tumor antigens inhibits melanoma growth,[34] while delivery of IFNα plasmid inhibits growth of squamous cell carcinomas.[35]

In the experiments described here, the intratumor delivery of plasmid DNA into B16 mouse melanomas by two different electroporation protocols was compared. These two types of electroporation conditions had been demonstrated previously for delivery of plasmid DNA into mouse melanomas. These protocols differed significantly in pulse length, field strength, and the electrode used for delivery (Figure 1). Relative reporter gene expression after intratumor plasmid delivery was first compared. Both protocols resulted in significantly increased plasmid expression, although EP1 increased reporter expression 21-fold, while EP2 increased expression 42-fold.

The effect on tumor growth of electroporation alone, luciferase plasmid injection alone, or the combination was observed in B16 mouse melanomas (Figure 2a). Tumors were treated as indicated on day 0, when they were approximately 4 mm in diameter, day 3, and day 7. Application of pulses after injection of saline only or injection of 100 μg plasmid DNA without pulses only minimally slowed tumor growth. Interestingly, when plasmid injection was combined with intratumor electroporation, a significant number of complete tumor

Correspondence: LC Heller, University of South Florida, Center for Molecular Delivery, c/o Department of Surgery MDC16, 12901 Bruce B Downs Boulevard, Tampa, FL 33612, USA
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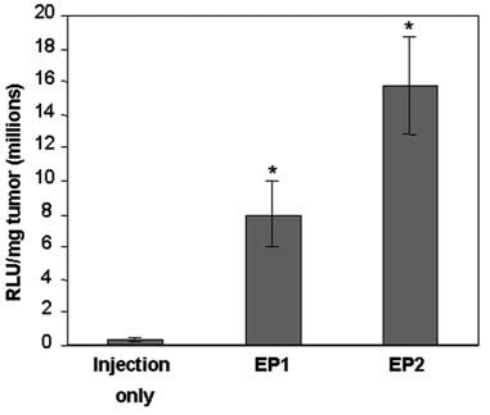

Figure 1 *1 Reporter expression after intratumor delivery of a plasmid encoding luciferase. Female C57Bl/6b mice were injected subcutaneously in the left flank with 10^6 trypsinized B16.F10 (ATCC CR6475) mouse melanoma cells in 50 μl sterile injectable saline using an 0.5 inch 30-gauge needle. Tumors were allowed to grow 8 days to a mean diameter of approximately 4 mm before treatment (day 0). Mice were anesthetized during all treatments using a mixture of 2.5% isoflurane and 97.5% O_2. Tumors were then injected with 50 μl 2 μg/μl plasmid DNA (VR1255, Vical, San Diego, CA, USA) prepared using an endotoxin-free method (Qiagen, Valencia, CA, USA). Endotoxin levels were confirmed to be less than 0.1 EU/μg (QCL-1000, Biowhittaker, Walkersville, MD, USA). One of two electoporation protocols, consisting of either six 100-μs pulses at a field strength of 1500 V/cm and a frequency of 1 Hz with a six needle array (EP1[36]) or 10 5-ms 800 V/cm pulses at a frequency of 1 Hz delivered with caliper electrodes moistened with electrode paste (EP2[25]), was immediately applied using a T820 Electrosquare porator and autoswitcher (BTX, San Diego, CA, USA). After 24 h, tumors were assayed for luciferase activity. Mice were humanely killed, and tumors removed, weighed, and homogenized in 25 mM Tris, pH 7.8, 2 mM DTT, 2 mM EDTA, pH 8.0, 10% glycerol. Extracts were assayed for luciferase activity[37] using a MLX microtiter plate luminometer (Dynex Technologies, Chantilly, VA, USA). Data are reported as relative light units per milligram tumor tissue. Bars represent the mean and standard error of the mean from three independent experiments, each containing three to four tumors per treatment group. Statistical significance ($P < 0.05$), relative to injection alone is noted by an asterisk.*

regressions were observed when using one, but not the other electroporation protocol, even though these two delivery methods resulted in only a two-fold difference in reporter expression. This regression occurred after injection of plasmid DNA followed by electroporation protocol EP2, but not after the application of electroporation protocol EP1. The observed regression was long-lived and occurred in a significant number of animals ($P < 0.05$, Figure 2b). This effect might have been related to the amount of DNA delivered intracellularly for two reasons. First, it was associated with the electroporation protocol that resulted in the higher level of plasmid expression as demonstrated by reporter gene expression (Figure 1). Second, this effect displayed a dependence on DNA concentration, since sustained complete regression occurred in 42% of tumors electroporated with 50 μg plasmid DNA, and this percentage increased to 83% in tumors electroporated with 100 μg plasmid DNA.

Because of this interesting observation, the remaining experiments focused on DNA delivery using electroporation protocol EP2. This antitumor effect was neither plasmid-dependent nor dependent on the presence of eukaryotic coding sequences. When melanomas were treated three times with electroporation with the plasmid pUC18, the effect on tumor growth was confirmed (Figure 3a).

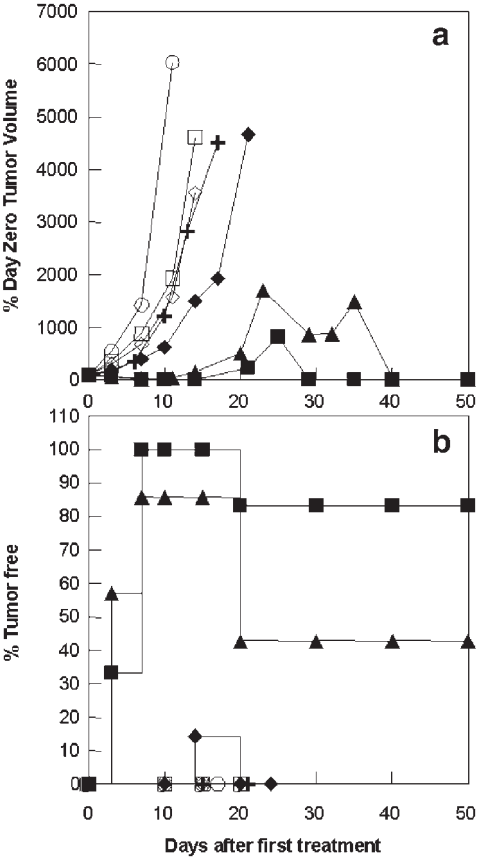

Figure 2 *The effect of three intratumor electroporation deliveries of plasmid DNA on tumor volume. Tumors were induced as described in Figure 1. After tumors grew to a mean diameter of 4 mm (day 0), tumors were treated with VR1255 and electroporation as in Figure 1 on days 0, 3, and 7. Tumors were then measured twice weekly using a digital caliper. Tumor volume was calculated by the standard formula $v = ab^2/6$, where a is the longest diameter, and b is the next longest diameter perpendicular to a. In the case of continued tumor growth or tumor recurrence, the animal was considered incurable and humanely killed when the tumor volume reached 1000 mm^3. Each individual tumor volume was normalized to its volume on day 0, the first day of treatment. (a) Tumor volumes and (b) tumor-free animals after delivery of plasmid DNA (VR1255) with electroporation. ○, no treatment; +, injection of 100 μg pDNA only; ◇, saline injection followed electroporation protocol EP1; □, saline injection followed by electroporation protocol EP2; ◆, injection of 100 μg pDNA followed by EP1; ▲, injection of 50 μg pDNA followed by EP2, ■, injection of 100 μg pDNA followed by EP2 (n = 6–7 mice).*

The regression induced by this treatment was also long-lived (Figure 3b), with approximately 70% of mice surviving tumor-free until challenge. For the challenge, all mice in the experiments described which remained tumor-free were injected along with naive controls on the opposite flank with 5×10^5 B16 cells at day 56, 63, or 74 and monitored for another 50 days. While visible tumors appeared within 10 days in all the naive control mice, 70% (12 of 17) of mice did not grow challenge tumors and so may have developed immunity to B16 cells.

The sustained complete regression observed was also dependent on the number of treatments applied. When only a single treatment of 100 μg plasmid DNA followed by electroporation was used, five of six tumors completely regressed, but four of these tumors recurred by day 14 (data not shown). The remaining tumor did not

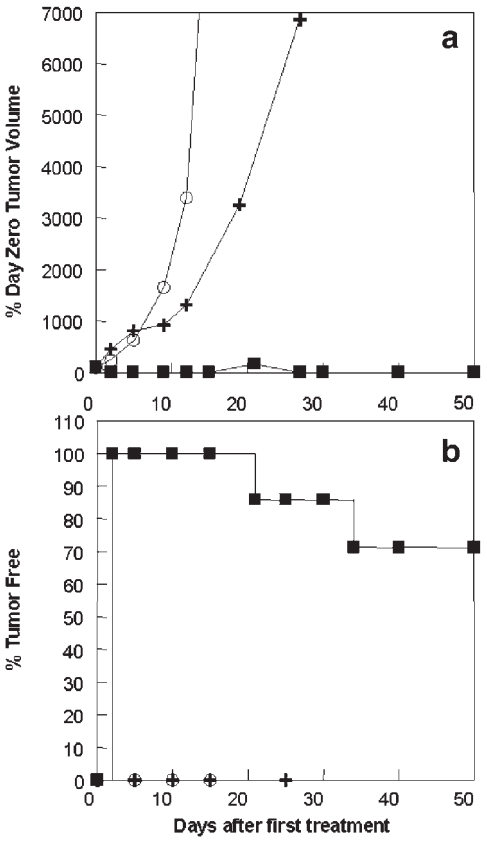

Figure 3 *Eukaryotic coding sequences are not necessary for the antitumor effect. (a) Tumor volumes and (b) tumor-free animals after delivery of 100 μg plasmid DNA (pUC18) using electroporation protocol EP2 on days 0, 3, and 7. ○, no treatment; +, injection of 100 μg pDNA only; ■, injection of 100 μg pDNA followed by electroporation (n = 6–7).*

recur, but the mouse succumbed to subcutaneous challenge with B16 melanoma cells.

Since this effect was not dependent on expression of a therapeutic protein, it might have been due to a response to the bacterially derived DNA itself. In vertebrates, the dinucleotide CpG occurs at about one fifth the expected frequency and 60–90% of those motifs are altered to 5′methylcytosine.[38] Unmethylated bacterial CpGs act as a signal for innate immunity in mammals. The immunostimulatory effect of unmethylated CpGs can be observed after uptake of bacterial genomic DNA, oligonucleotides,[39] or plasmid DNA.[40] pUC18 contains 156 copies of the dinucleotide CpG, including 20 copies of a six base immunostimulatory consensus sequence.[41] Immunostimulatory DNA has been described as an anticancer immune adjuvant, originally with *Mycobacterium bovis* genomic DNA.[42,43] When CpG motif oligonucleotides are injected with a lymphoma surface antigen, a specific antilymphoma immune response is generated.[44] When the animals were challenged with lymphoma cells, survival improved when compared with control mice. The addition of treatment with soluble GM-CSF increased this survival.[45] Intravenous injection of lipid-CpG motif plasmid DNA complexes reduced the lung tumor burden in mice injected 3 days before treatment with MCA-205 fibrosarcoma, B16.F10 melanoma, and CT26 colon carcinoma cells.[46]

To explore this antitumor effect, tumors were excised and examined histologically 24 h after treatment, when most tumors began regressing (Figure 4). Untreated tumors exhibited a few inflammatory cells. Injection of DNA alone induced 5–20% necrosis and tumors were surrounded by a mixed inflammatory infiltrate. This is similar to the effect observed after muscle injection of plasmid DNA.[47] Injection of saline followed by electroporation resulted in skin burning associated with 10–50% apoptotic cells with areas of necrosis, and moderate infiltration of PMNs, macrophages, and lymphocytes. With injection of plasmid DNA followed by pulsing (Figure 5), the skin was again burned, but apoptosis increased to 80–100% of cells. Apoptosis was detected by TUNEL assay and semiquantitatively quantified as the percentage of apoptotic tumor cells related to the total number of tumor cells. The apoptotic tumor cells were clearly identified by their dark brown nuclei. Some nuclear chromatin condensation and/or fragmentation was also visible on the H&E stain (Figure 5a–d). The tumor became very congested and a strong infiltrate containing PMNs, macrophages, and some lymphocytes was observed. This indicated that this tumor regression might be due not only to induction of apoptosis, but also to a concomitant immune response. The presence of marked macrophagic and lymphocytic infiltration around and within the tumors exhibiting complete response supports this view. Furthermore, while some apoptosis was induced by pulses alone, pulses alone were not an effective antitumor therapy (Figure 2a and b). Combination with plasmid DNA was required.

Future experiments will confirm whether this anti-

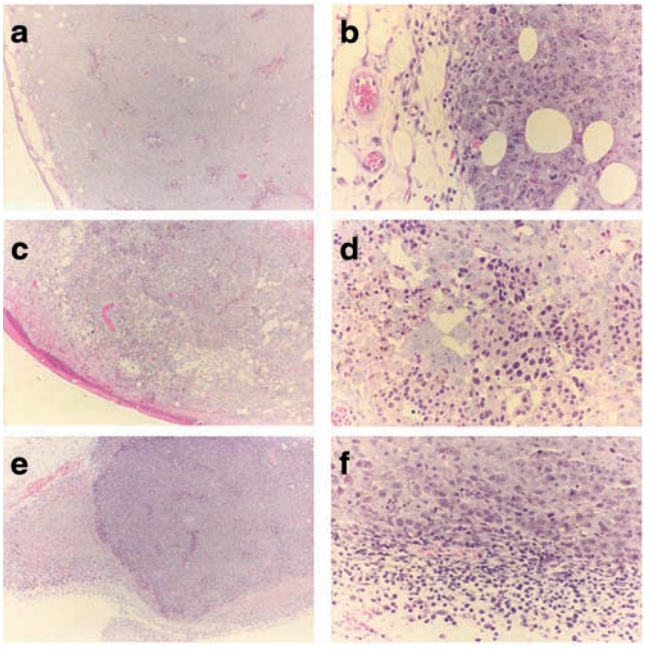

Figure 4 *Histological analysis of paraffin-embedded sections by hematoxylin and eosin (H&E) staining 24 h after treatment. Specimens from mouse melanoma tumors were fixed in 10% neutral buffered formalin for 6 h. After fixation, the tissue samples were processed into paraffin blocks. Four micrometer-thick tissue sections were obtained from the paraffin blocks and stained with hematoxylin and eosin (H&E, Richard-Allan Scientific, Kalamazoo, MI, USA) using standard histologic techniques. (a) Untreated tumor, ×40; (b) untreated tumor, ×250; (c) injection of 100 μg VR1255 only, ×40; (d) injection of 100 μg VR1255 only, ×250; (e) saline injection followed by electroporation protocol EP2, ×40; (f) saline injection followed by electroporation protocol EP2, ×250.*

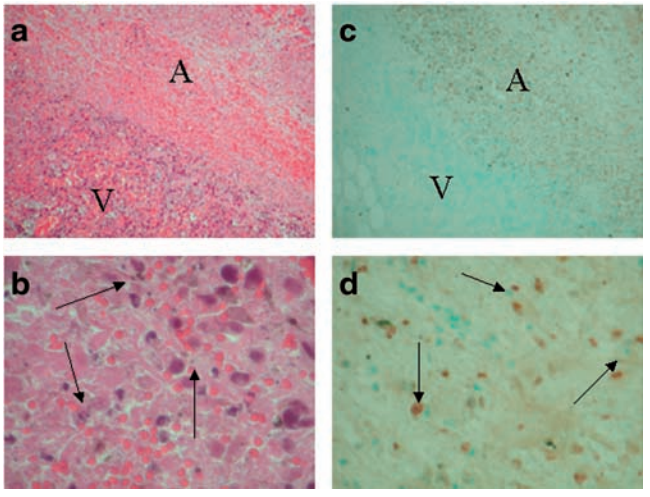

Figure 5 *Histological analysis of paraffin-embedded sections by hematoxylin and eosin (H&E) and TUNEL staining 24 h after delivery of 100 μg plasmid DNA (VR1255) using electroporation protocol EP2. Specimens from mouse melanoma tumors were bisected and half frozen at −70°C, and half was fixed in 10% neutral buffered formalin for 6 h. After fixation, the tissue samples were processed into paraffin blocks. Four micrometer-thick tissue sections were obtained from the paraffin blocks and stained with hematoxylin and eosin (H&E, Richard-Allan Scientific) using standard histologic techniques. Apoptosis was determined by TdT-mediated dUTP nick end labeling (TUNEL) using in situ cell death detection kit (Boehringer Mannheim). Frozen sections were prepared from the frozen tissues. The slides were fixed in paraformaldehyde (4% in PBS, pH 7.4). After rinsing with PBS and incubation in permeabilization solution, the tissues were cross reacted with TUNEL reaction mixture (for 60 min at 37°C in a humidified chamber), with converter–alkaline phosphatase solution (for 30 min at 37°C in a humidified chamber), and with alkaline phosphate substrate solution (Vector Laboratories, Burlington, MA, USA) (for 5 to 10 min). The reactions were analyzed by light microscopy. (a) H&E, ×100; (b) H&E, ×600; (c) TUNEL, ×100; (d) TUNEL, ×400. A, apoptotic tumor cells; V, viable tumor cells, arrows indicate apoptotic cells (brown-stained cells on the TUNEL assay).*

tumor effect is due to the immune response to CpG motif DNA or to some other factor. In addition, the observed induction of apoptosis will be explored.

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