



Published in final edited form as:

Cancer Gene Ther. 2013 December ; 20(12): 695–700. doi:10.1038/cgt.2013.71.

Electrotransfer of single-stranded or double-stranded DNA induces complete regression of palpable B16.F10 mouse melanomas

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Abstract

Enhanced tumor delivery of plasmid DNA with electric pulses *in vivo* has been confirmed in many preclinical models. Intratumor electrotransfer of plasmids encoding therapeutic molecules has reached Phase II clinical trials. In multiple preclinical studies, a reduction in tumor growth, increased survival, or complete tumor regression have been observed in control groups in which vector or backbone plasmid DNA electrotransfer was performed. This study explores factors that could produce this antitumor effect. The specific electrotransfer pulse protocol employed significantly potentiated the regression. Tumor regression was observed after delivery of single-stranded or double-stranded DNA with or without CpG motifs in both immunocompetent and immunodeficient mice, indicating the involvement of the innate immune system in response to DNA. In conclusion, this study demonstrated that the observed antitumor effects are not due to a single factor, but to a combination of factors.

Keywords

Electroporation; electrotransfer; melanoma; tumor regression

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Author conflicts of interest:

Authors declare no competing financial interests in relation to the work described.

Introduction

Therapeutic gene delivery using electric pulses (electrotransfer) has been achieved in a variety of tissue types including skin, kidney, liver, testis, brain, cartilage, arteries, prostate, cornea, and skeletal muscle^{1, 2}. Intratumor electrotransfer of plasmids encoding therapeutic genes has been developed in preclinical models^{3, 4} and has been used in veterinary oncology⁵ and clinical trials⁶.

Interestingly and apparently independent of specific genes, inhibition of tumor growth, increased survival time, and complete tumor regression have been observed after intratumor electrotransfer of control plasmids, either plasmid devoid of only therapeutic gene or plasmids encoding reporter genes to palpable tumors in preclinical models. These effects have been described in melanomas^{7–11}, lung carcinomas^{12, 13}, fibrosarcomas¹⁴, pancreatic carcinomas¹⁵, mammary tumors¹⁶ and colorectal carcinomas^{17, 18}. In two studies, antitumor effects were observed after intratumor electrotransfer of an oligonucleotide containing a CpG motif, although each oligonucleotide primarily functioned as an antisense molecule^{19, 20}. The large number of variables between these studies limits the ability to explore the mechanism of this non-specific tumor regression.

This study explored potential factors that may play a role in this observed antitumor effect in the well-characterized mouse B16.F10 melanoma tumor model. Intratumor plasmid electrotransfer by four electroporation pulse protocols that varied considerably in pulse number, length, and field strength was compared^{12, 21–23}. Previous studies confirmed that application of pulses alone did not induce tumor regression^{8, 12, 23}; combination with DNA is required. Therefore, the DNA composition was also explored.

Materials and Methods

Tumor and animal models

All procedures were approved by the Institutional Animal Use and Care Committee of the University of South Florida College of Medicine, Tampa, FL, USA (#R2736) and Veterinary Administration of The Ministry of Agriculture and the Environment of the Republic of Slovenia (#34401-12/2009/6). One million B16.F10 (ATCC CR6475) mouse melanoma cells in 50 µl PBS were injected subcutaneously in the left flank of female 7–8 week old C57Bl/6 (Jackson Laboratories, Bar Harbor, ME) or SCID (Harlan, Udine, Italy) mice. Tumors were allowed to grow approximately eight days to an approximate diameter of four mm before manipulation.

Plasmids and oligonucleotides

Plasmids gWizLuc and gWizBlank (Aldevron, Fargo, ND) were commercially prepared (Aldevron, Fargo, ND). Oligonucleotides were modified by the inclusion of phosphorothioate internucleotide bonds (IDT DNA, Coralville, IA or Invivogen, San Diego, CA) to produce nuclease resistance. Oligonucleotide sequences were ODN 1668, 5'-TCCATGACGTTCCCTGATGCT-3' and ODN 1720, 5'-TCCATGAGCTTCCTGATGCT-3'^{24, 25}. All molecules were suspended in sterile injectable saline at 2 mg/ml.

Electrotransfer in vitro

Confluent low passage B16.F10 cells were prepared in electroporation buffer at a concentration of 25×10^6 cells/mL²⁶. Cells (80 μ L of final cell suspension) were mixed with 20 μ L of gWizBlank plasmid (1 mg/mL). One half of the mixture was placed between 2 mm gap steel electrodes and exposed to 8 square wave electric pulses (electric field intensity 600 V/cm, pulse duration 5 ms, frequency 1 Hz). The other half of the mixture served as control for plasmid exposure without the electric pulses. Electric pulses were generated by in-house build electroporator (University of Ljubljana, Faculty of Electrical Engineering, Slovenia). After the application of electric pulses, the cells were incubated at room temperature for 5 min and then plated for cell viability assay. The number of viable cells was determined by PrestoBlue Cell Viability Reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol every 24 hours for four consecutive days. Cell proliferation rate was quantified by the slope of the linear regression between \log_{10} (cell proliferation) and time.

Electrotransfer in vivo

Deliveries were performed on day 0 (mean tumor diameter 4 mm), day 3, and day 7. Mice were anesthetized using a mixture of 2.5% isoflurane and 97.5% O₂. Tumors were injected with 50 μ L of molecule solution and pulsed immediately with caliper electrodes moistened with electrode paste as described in each figure at a frequency of 1 Hz using a T820 ElectroSquare porator (BTX Molecular Delivery Systems, Holliston, MA) or a Betatech Electro cell B10 porator (Betatech, Toulouse, France). Tumors were measured twice to three times weekly using a digital caliper. Tumor volume was calculated by the standard formula $v=ab^2\pi/6$, where a is the longest diameter, and b is the next longest diameter perpendicular to a . Any animal was considered incurable and humanely euthanized when the tumor volume reached 1000 mm³ or when the animal's behavior indicated discomfort. Animals with tumors in regression were followed up to 100 days after the first electrotransfer. After that, if no tumor regrowth was observed, animals were considered to be in complete regression.

Statistical analysis

Comparison of complete long-term regression levels between groups was performed using Holm Sidak test after one way ANOVA was performed and fulfilled or Dunnett's Method after Kruskal-Wallis One Way Analysis of Variance on Ranks (SigmaPlot, Systat, San Jose, CA).

Results

When plasmid DNA was electrotransferred into B16.F10 cells *in vitro*, a small but significant decrease in viability was observed (Figure 1a). This effect was not observed with plasmid addition or pulse application alone. Cell proliferation rate was calculated for each treatment group, and a significantly reduced cell proliferation rate was observed after plasmid DNA electrotransfer. Cell proliferation rate was not affected by plasmid exposure or pulse application alone (Figure 1b).

To determine the relative importance of the electrotransfer pulse protocol to the non-specific antitumor effect observed *in vivo*, tumor regression after vector plasmid delivery with four well-characterized electrotransfer protocols was assessed (Figure 2). Delivery of ten 5 ms pulses at a field strength of 800 V/cm, generated significant long-term tumor regression ($p < 0.05$) in 25% of animals. Two other protocols, eight 20 ms pulses at a field strength of 500 V/cm and one 100 ms pulse at a field strength of 667 V/cm also induced a lesser but statistically significant regression levels ($p < 0.05$). In contrast, application of six 50 ms 150 V/cm pulses did not induce tumor regression.

CpG motif DNA is a well-characterized immunostimulatory agent in the plasmid sequence that may be responsible for the observed antitumor effect²⁴. Control and CpG oligonucleotides were delivered to determine if DNA containing this motif would induce regression of melanomas in immunocompetent C57Bl/6 (Figure 3a) and SCID mice (Figure 3b). In immunocompetent C57Bl/6 mice, electrotransfer of the Type B oligonucleotide (ODN) 1668^{24, 25}, containing a mouse specific motif (GACGTT)²⁷, induced significant long-term and complete tumor regression in 36% of animals ($p < 0.05$), while simple injection did not. Injection or electrotransfer of control ODN 1720, lacking this motif, did not induce regression. Intriguingly, electrotransfer induced long-term and complete tumor regression ($p < 0.05$) in 20% of immunodeficient SCID animals receiving ODN 1668 (CpG motif) oligonucleotide and 40% of animals receiving control ODN 1720 (GpC motif) oligonucleotide. In addition, short term tumor regression was induced by electrotransfer of either oligonucleotide, apparently resulting in significant tumor growth delay ($p < 0.05$), being 15.7 ± 6.0 days after electrotransfer of Type B ODN 1668 and 8.5 ± 2.0 days after electrotransfer of control ODN 1720.

The typical appearances of tumors treated with electrotransfer of either of oligonucleotides in SCID mice are shown in Figure 4. When long-term tumor regression was achieved 100 days after electrotransfer of either of the oligonucleotides, only minimal scarring was observed at the site of electrode position, either completely without residual pigmentation or with small residual pigmentation at the site of tumor transplantation (Figs. 4 a, b). When only short-term tumor regression was achieved, the newly formed tumors regrew from the treated area (Figs. 4c, d). The short term regression was present for longer time in the group of mice that received Type B ODN 1668 (from 2–27 days) compared to mice that received control ODN 1720 (2–14 days).

Discussion

This study explored potential factors that may play a role in the antitumor effects observed in many preclinical studies after intratumor electrotransfer of control plasmids. *In vitro*, viability of B16.F10 cells and their proliferation rate were significantly reduced by plasmid DNA electrotransfer, which was not observed after exposure to plasmid DNA or electric pulses alone. Reduction of cell viability after application of electric pulses was expected as it was demonstrated previously that long electric pulses at low electric field strength that were used in the *in vitro* experiment affect cell viability²⁸. Since cell proliferation and metabolism are interconnected via common regulatory pathways²⁹, it is possible that the decrease in cell proliferation rate is a reflection of changes in cellular metabolism in response to plasmid

DNA. However, this decrease in cell proliferation and viability was not likely to explain the complete tumor regression observed *in vivo*.

Different electrotransfer pulse protocols delivering an identical lot and dosage of plasmid DNA produced different levels of tumor regression. This regression was not related to the quantity of plasmid successfully delivered as demonstrated by reporter expression³⁰ or to the pulse energy^{31, 32}, pulse number, pulse intensity or the pulse length. In groups where regression was observed, a relationship between regression and pulse field strength ($R^2 = 0.9976$) was observed. The rationale behind this strong association is not clear. However, the pulse regimen was a significant variable in the generation of tumor regression.

Another possible factor involved in the antitumor effect of control plasmids is the presence of CpG motifs²⁴ in the plasmid sequence. These motifs are bound by the endosomal toll-like receptor 9 (TLR9)³³, which is found primarily in dendritic and B cells, producing immune stimulation. The immune stimulation by CpG motif DNA has been utilized as a monotherapy or in combination therapies in clinical trials for cancer therapies and as vaccine adjuvants^{34, 35}. In animals with palpable B16.F10 tumors, a survival increase and tumor growth inhibition was observed after multiple intratumoral³⁶ or peritumoral³⁷ injections of the Type B ODN 1826, which contains two mouse-specific CpG motifs. Significantly prolonged survival and tumor growth inhibition were observed in this model when intraperitoneal injection of Type A ODN 1585 was initiated simultaneously with intraperitoneal tumor cell injection³⁸. Complete tumor regression was not described in these studies.

In the study described here, electrotransfer of Type B ODN 1668 produced a significant antitumor effect in immunocompetent mice and a less pronounced effect in SCID mice. This difference is likely a consequence of activation of different signaling pathways involved in the stimulation of immune system³⁹. Specifically, type B CpG oligonucleotides induce strong B cell activation and moderate NK cell activation⁴⁰. SCID mice lack functional B-cells⁴¹, thus no response to B-cell mitogens is expected. However, to some extent, type B CpG oligonucleotides can activate NK cells^{42, 43}. Hence, the observed antitumor effect in immunodeficient mice could be mediated through activation of NK cells. However, this should be confirmed by analysis of cytokine production in response to CpG ODNs in both types of mice.

In SCID mice, tumor regression was also observed after electrotransfer of the control oligonucleotide. Although both CpG and control oligonucleotides induced complete tumor regression in SCID mice, differences were observed between the two groups. Short-term tumor regression was more pronounced in mice receiving electrotransfer of CpG oligonucleotides, all mice in the group were tumor free for up to 12 days, while only 70% of mice were tumor free for up to 12 days after electrotransfer of control oligonucleotides. On the other hand, tumors regressed in only 20% of SCID mice after electrotransfer of CpG oligonucleotides as opposed to 40% after electrotransfer of control oligonucleotides. In previous studies, liposomal-mediated transfection of different oligonucleotides was used for determination of TLR9 activation. The authors demonstrated activation in response to oligonucleotides independently of the CpG motif and regardless of their methylation

status^{44, 45}. A switch from CpG to GpC motif in oligonucleotides creates a low affinity ligand for TLR9⁴⁶ and with natural DNA uptake pathway into the cells their concentration is too low to activate TLR9. These results support the hypothesis that upon enhanced endosomal translocation, in our case by electrotransfer, low affinity ligands in endosomes can reach the threshold concentrations required to drive TLR9 activation^{44, 45}. Consequently, NK cells are activated and may exert antitumor effectiveness, which was observed in SCID mice. In any case, tumor regression was observed in SCID mice, so specific cellular and humoral immunity was not required. The lack of tumor response in immunocompetent mice after control ODN electrotransfer and its underlying mechanisms require further experimental studies.

In addition to TLR9 receptors, intracellular DNA can be recognized by several cytoplasmic double-stranded DNA sensors which, when bound to DNA, activate cascades producing inflammation and programmed cell death⁴⁷. The specific ligands for these cytosolic sensors are not well characterized and may be redundant⁴⁸. Similar to TLR9, these sensors regulate type I interferon production and mediate inflammatory responses⁴⁹. DNA sensors are compartmentalized within the cell, and electrotransfer may be uniquely situated to activate both endosomal and cytosolic DNA sensors. Electrotransfer is primarily an endocytosis-driven process^{50, 51} although DNA is observed in aggregates and in free form in the cytoplasm after electrotransfer^{51, 52}. The half-life of cytosolic free DNA is short and probably is a dead end process as far as gene expression is involved⁵³. However, its presence may be adequate to activate cytoplasmic DNA sensors. This DNA compartmentalization after electrotransfer may also explain the lack of correlation between tumor gene expression and tumor regression³⁰. The production of pro-inflammatory and chemokines is observed in tumors³⁰ and in muscle⁵⁴ after DNA electrotransfer. This upregulation supports the concept that endosomal and/or cytosolic nucleic acid receptors, potentially including both TLR9 and cytosolic DNA sensors, are activated, generating an antitumor immune response.

In conclusion, our study confirmed sporadic observations in multiple preclinical tumor models of non-specific tumor regression after control vector electrotransfer in a single tumor type and single vector, including its concentration, batch, and diluent. The antitumor effects after electrotransfer of control vector plasmid DNA were due to multiple factors. Tumor regression was influenced by the pulse regimen, although the presence of DNA was required. Sequence motifs in DNA sequences contributed to tumor regression. Still, tumor regression was observed also in SCID mice after delivery of both CpG and GpC oligonucleotides, indicating that innate immune system was likely activated through signaling pathways of multiple nucleic acid sensors. A basic understanding of the mechanism by which this regression occurs is important in any tumor electrotransfer therapy. The knowledge that additional signaling pathways are induced is important in therapeutic design as it may aid or hinder immune-based or other targeted cancer therapies. In delivery to other tissues, this understanding will also be important, both in the prediction of the therapeutic outcome after specific gene delivery, and, if necessary, in designing method to avoid pathway activation in situations where this induction is not desirable.

Acknowledgements

The work in this study was supported by NIH 1 R21 CA106860 (LH), by the Slovene Research Agency P3-0003 and J3-4259 (MC and VT), and by a Bilateral scientific cooperation between Republic of Slovenia and United States of America BI-US/11-12-011.

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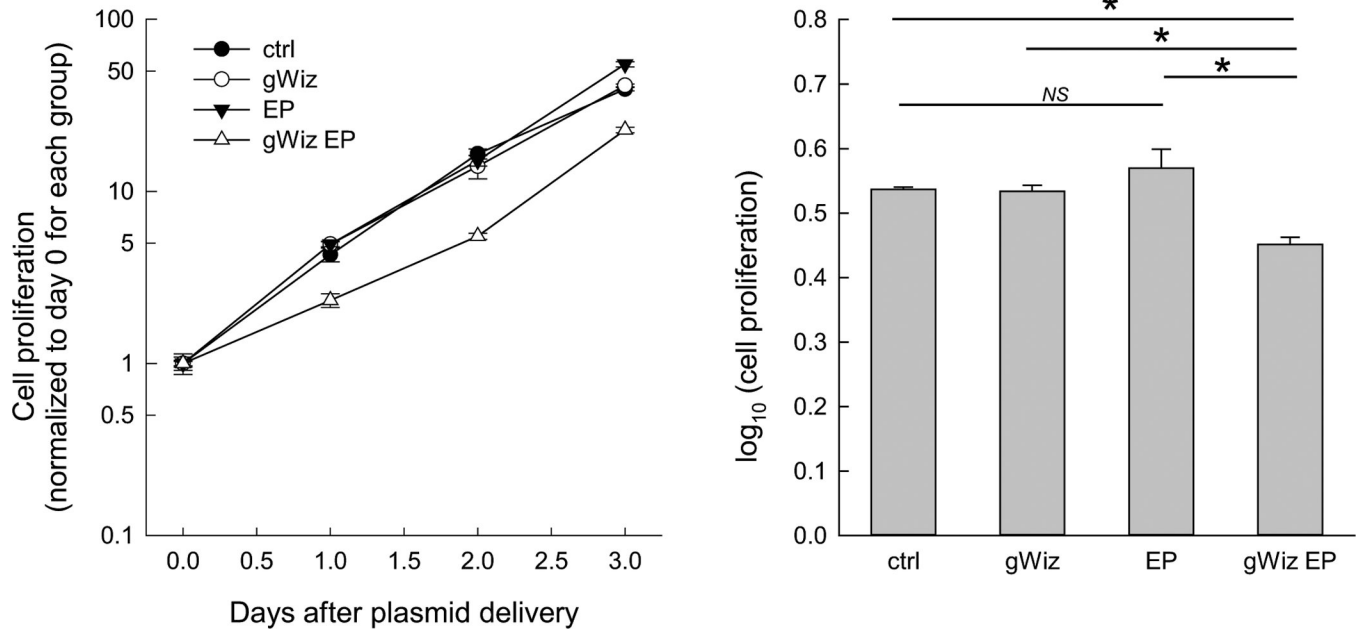


Figure 1. Effect of DNA electrotransfer on cell proliferation *in vitro*

(a) Electrotransfer of gWizBlank plasmid induced a significant decrease in viability, that was not observed by plasmid alone or application of electric pulses alone. 20 μ g of plasmid (1 mg/mL) was mixed with 2×10^6 B16.F10 cells and half of this mixture was exposed to eight 5 ms 600 V/cm electric pulses with a plate electrode, whereas the other half served as control for plasmid alone. Open circles, untreated; filled circles, electroporation; open triangles, gWizBlank without pulses; filled triangles, gWizBlank with pulses. ANOVA two sided, $P < 0.05$ * statistical significance. (b) Electrotransfer of gWizBlank plasmid induced a significant decrease in cell proliferation rate in comparison to control cells, that was not observed by plasmid alone or application of electric pulses alone. Bars: mean cell proliferation rate \pm st. error of the mean. One way ANOVA, $P < 0.05$ * statistical significance, NS not significant.

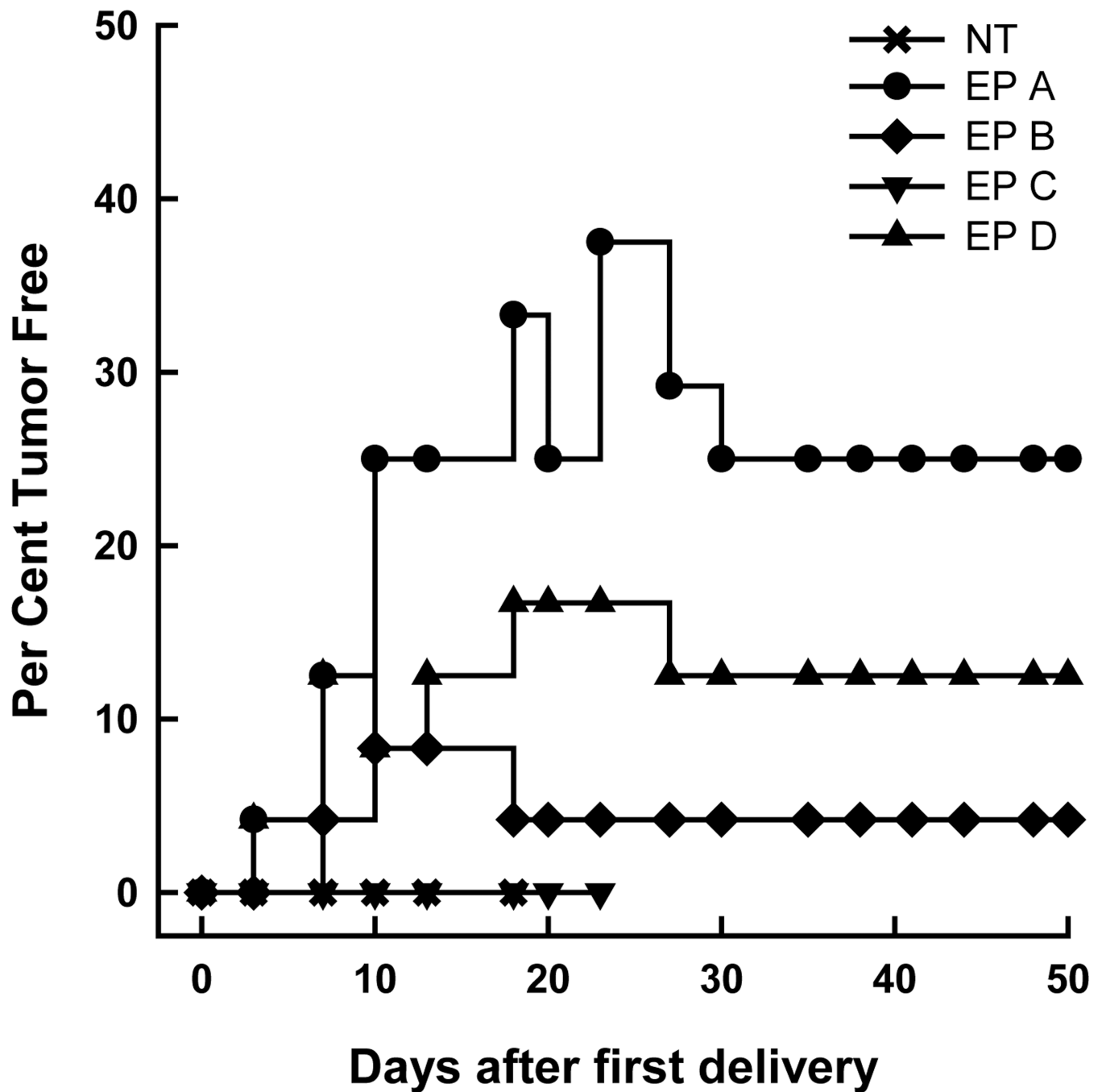


Figure 2. Long-term tumor regression after electrotransfer using several pulse protocols
 100 μ g commercially prepared gWizBlank was delivered by electrotransfer into palpable melanoma tumors in the flanks of C57Bl/6 mice using various pulse protocols: X, untreated; filled circles, ten 5 ms 800 V/cm pulses with a caliper electrode (EP A)²¹; filled diamonds, eight 20 ms 500 V/cm pulses with a caliper electrode (EP B)¹²; filled inverted triangles, six 50 ms 150 V/cm pulses with a caliper electrode (EP C)²²; filled triangles, one 100 ms 667 V/cm pulse with a caliper electrode (EP D)²³. Tumor growth or regression was monitored as described in methods. n=24 for all groups.

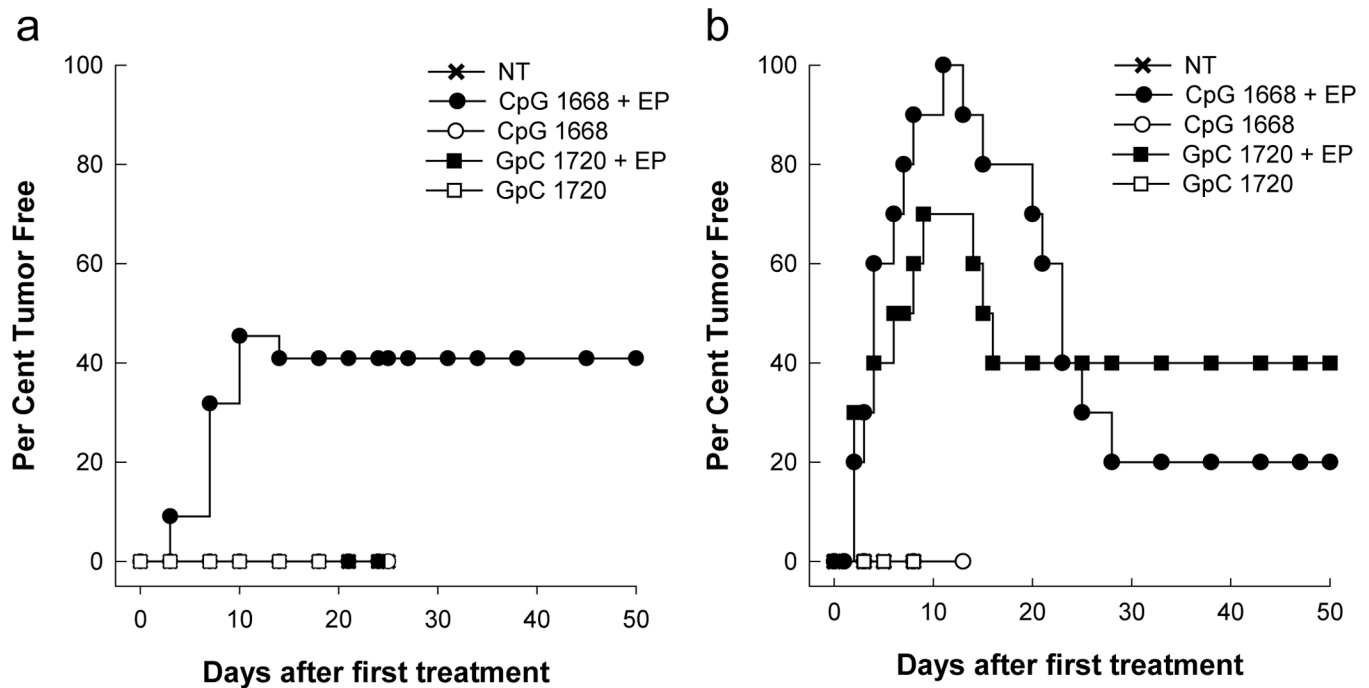


Figure 3. Long-term tumor regression after electrotransfer of CpG motif oligonucleotides
 100 μ g oligonucleotide was injected with or without pulses into palpable melanoma tumors using ten 5 ms 800 V/cm pulses with caliper or plate electrode three times in one week. (a) C57Bl/6 mice. X, untreated (n=16); filled squares, CpG ODN 1668 with pulses (n=15); open squares, CpG ODN 1668 without pulses (n=6); filled triangles, GpC ODN 1720 with pulses (n=15); open triangles, GpC ODN 1720 without pulses (n=6). (b) SCID mice, identical symbols, n=10 per group. Tumor growth or regression was monitored as described in methods.

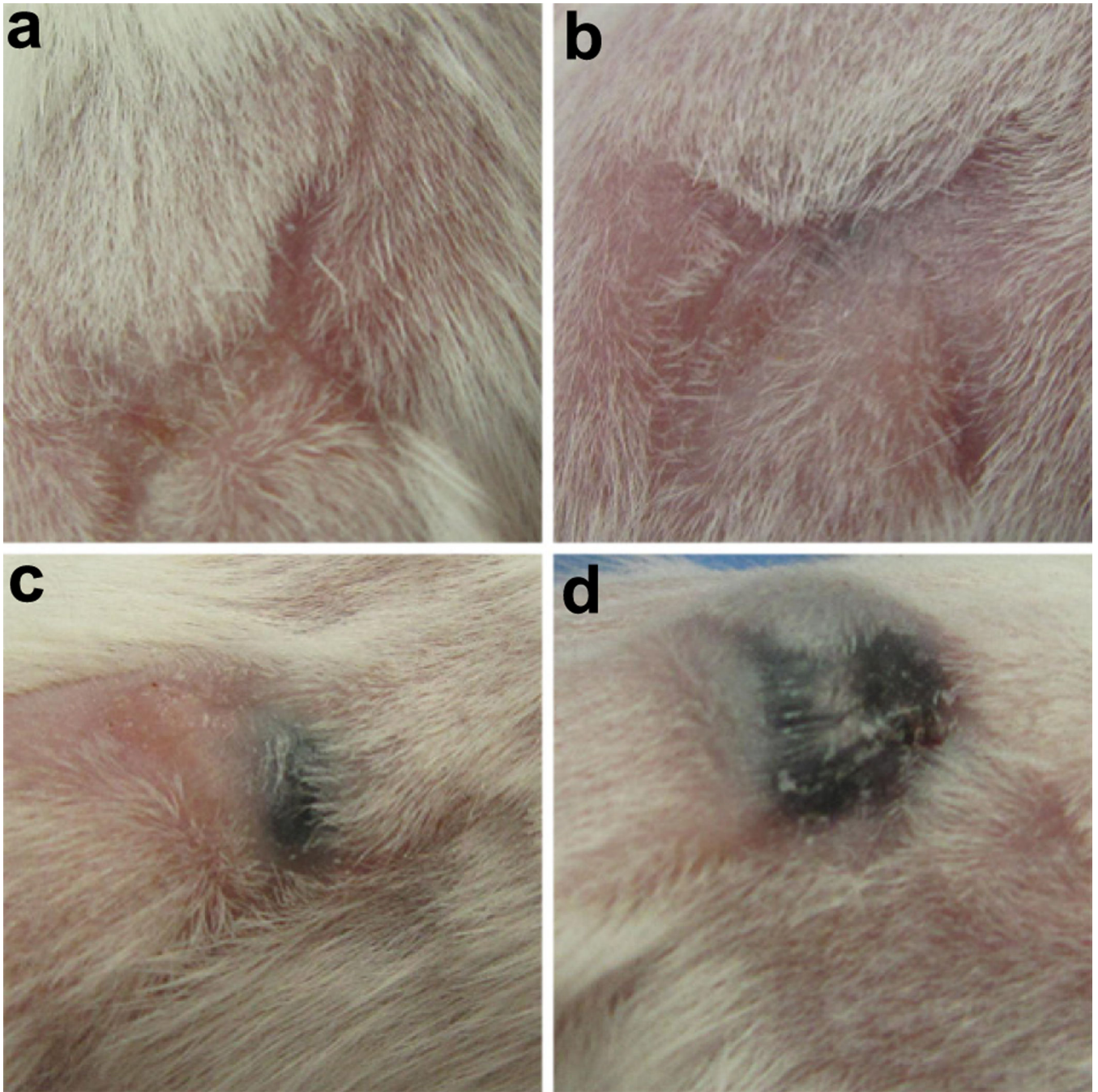


Figure 4. Tumor regression after electrotransfer of CpG motif oligonucleotides in SCID mice
On day 23 after first electrotransfer of oligonucleotides using ten 5 ms 800 V/cm pulses with a plate electrode, different outcomes were observed: (a) minimal scarring at the tumor site after electrotransfer of Type B ODN 1668 and (b) electrotransfer of control ODN1720, where pigmentation at the site of tumor regression is still present; (c) tumor regrowth at the tumor site after electrotransfer of Type B ODN 1668; and (d) tumor regrowth was quicker after electrotransfer of control ODN 1720.