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Enhancement of Electric Field-Mediated Gene Delivery Through Pretreatment of Tumors With a Hyperosmotic Mannitol Solution

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Abstract

Pulsed electric fields can enhance interstitial transport of plasmid DNA (pDNA) in solid tumors. However, the extent of enhancement is still limited. To this end, effects of cellular resistance to electric field-mediated gene delivery were investigated. The investigation used two tumor cell lines (4T1 and B16.F10) either in suspensions or implanted in two *in vivo* models (dorsal skin-fold chamber (DSC) and hind leg). The volume fraction of cells was altered by pretreatment with a hyperosmotic mannitol solution (1 M). It was observed that the pretreatment reduced the volumes of 4T1 and B16.F10 cells, suspended in an agarose gel, by 50% and 46%, respectively, over a 20-min period but did not cause significant changes *ex vivo* in volumes of hind leg tumor tissues grown from the same cells in mice. The mannitol pretreatment *in vivo* improved electric field-mediated gene delivery in the hind leg tumor models, in terms of reporter gene expression, but resulted in minimal enhancement in pDNA electrophoresis over a few micron distance in the DSC tumor models. These data demonstrated that hyperosmotic mannitol solution could effectively improve electric field-mediated gene delivery around individual cells *in vivo* through increasing the extracellular space.

Keywords

electric field-mediated gene delivery; in vivo electrophoresis; interstitial transport

Introduction

Electric field-mediated gene delivery is a physical method for improving efficiency of transfection with naked plasmid DNA (pDNA). This method has been shown to improve gene delivery *in vivo* in a variety of tissues including solid tumors 1–4. However, mechanisms behind the improved delivery are only partially understood since the majority of studies on electric field-mediated gene delivery in solid tumors have focused on the effects the parameters of the applied electric field (*e.g.*, electric pulse strength, duration, and

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Conflict of interest

The authors declare no conflict of interest.

number) had on quantifiable outputs, such as reporter gene expression and tumor regression 4.

Previous studies have shown that cell membrane electropermeabilization and pDNA electrophoresis are involved in electric field-mediated gene delivery. Electropermeabilization has been studied extensively *in vitro* 5, 6 and *in vivo* 7–10, and is the subject of many reviews 1–4, 10. The potential significance of *in vivo* electrophoresis on improved gene expression has been demonstrated by a series of studies conducted in muscle 11, 12 and solid tumors 13–17. These studies suggest that following an initial electropermeabilizing pulse, the electrophoretic movement of pDNA plays a determinant role in improved transgene expression. The magnitude of *in vivo* electrophoretic movement in tumor interstitium has been quantified directly both *ex vivo* 17, and *in vivo* 13–15. These studies concluded that an external electric field was able to increase pDNA mobility but the overall magnitude of movement with a 10-pulse sequence was less than the radius of a typical cell (~5 μm). The limited movement was attributed to physiological barriers formed by extracellular matrix (ECM) and cells in solid tumors. Previous studies have also shown that both concentration and structure of collagen but not glycosaminoglycans in ECM are the key factors effecting macromolecular transport in tumor interstitium 18–20. Therefore, collagen remodeling induced by a recombinant human relaxin (rh-Rlx) can enhance electric field-mediated gene delivery if it effectively reduces the collagen concentration in collagen-rich tumors 13.

The objective of this study was to investigate effects of cellular resistance to pDNA electrophoresis in tumors. The study used the same tumor models as those in the ECM study 13, so that the experimental data could be compared. It has been observed in these tumors that tumor and stromal cells make up approximately 33 % and 18 %, respectively, of the total tissue volumes 21. The cells are impermeable to pDNA and thus hinder pDNA movement in tissues. The hindrance can be reduced by decreasing the cell volume or increasing the interstitial space in tumors. In previous studies, the cell volume reduction has been achieved by treating tumors with apoptotic agents (e.g., paclitaxel) 22–24, which results in a significant decrease in tissue resistance to transport of macromolecules and nanoparticles. In this study, we investigated if the cellular resistance to pDNA transport could be reduced by treating tumors with a hyperosmotic solution of mannitol. Mannitol solution has been commonly used in the clinic to reduce intracranial pressure following traumatic head injury 25, or to treat patients with oliguric renal failure 26. It has also gained attention for its effectiveness in opening the blood brain barrier by shrinking endothelial cells in brain capillaries 27.

Effects of hyperosmotic solution of mannitol on tissue penetration of macromolecules in solid tumors have been investigated a previous study 28. The study revealed that the mannitol solution could significantly increase the extent of tissue penetration of the dextran molecule with molecular weight of 2,000,000 (2M), and that the penetration depth was correlated to the change in the available volume fraction (K_{AV}) of dextran 2M. The present study was designed to investigate whether the pretreatment of 4T1 and B16.F10 tumors with the hyperosmotic solution of mannitol would similarly increase the tumor interstitial space, and therefore the extent of pDNA transport *in vivo* since previous studies have shown that

the rate of interstitial transport depends strongly on the pore sizes in the extracellular matrix 29, 30. In the study, a series of *in vitro* and *ex vivo* experiments were first performed to determine the kinetics of tumor cell or tissue volume reduction following the mannitol treatment. Then, effects of mannitol treatment on reporter gene expressions *in vitro* and *in vivo* were quantified. Correlations between gene expression level and interstitial transport were also investigated using the methods developed previously 13.

Materials and Methods

Kinetics of Cell Shrinkage In Vitro and Ex Vivo

4T1 (a murine mammary carcinoma) and B16.F10 (a metastatic subline of B16 murine melanoma) cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml streptomycin, and 100 U/ml penicillin at 37°C, 95% air and 5% carbon dioxide. Cells were harvested from flasks with 0.25% trypsin/EDTA and rinsed with DMEM. Cells were spun for 2 min at 1500 rpm and resuspended in DMEM to a final concentration of 6.25×10^5 cells/ml.

Tumor cell responses to hyperosmotic solution of mannitol were determined as follows. 1% (w/v) agarose was dissolved in DMEM, mixed 1:1 (v:v) with 4T1 or B16.F10 cell suspension and poured into 35 mm petri dish. The sample was allowed to solidify in a 37°C incubator and transferred to a temperature-controlled stage on an inverted fluorescence microscope (Axiovert 135TV, Zeiss, Thornwood, NY, USA). 1.0 mL 1.0 M mannitol in DMEM was pipetted on top of the agarose gel with cells; and images were acquired every minute for 90 min. Image analysis software (Image-Pro Plus®, Media Cybernetics, Inc., Silver Spring, MD, USA) was used to calculate the area of individual cells at each time point. The area was then used to determine the average radius of the cell, from which the volume was calculated, assuming the cells to be spherical. Individual cell volumes were normalized by their initial values at $t = 0$. The experiment was repeated with five different samples; and the mean and the standard deviation of the data from 48 cells are reported in this paper.

Tumor tissue responses to hyperosmotic solution of mannitol were determined in a similar fashion. 4T1 and B16.F10 hind leg tumor models were prepared in female Balb/C and C57BL/6 mice, respectively, as described in 13, 21. Tumors were allowed to grow to 8–10 mm in diameter, and then the mice were sacrificed and the tumors were excised and cut into 1-mm sections. A 2-mm biopsy punch was used to cut circular pieces from the sections. These tumor biopsies were suspended in 1% w/v agarose in DMEM, and the sample was allowed to solidify in 35 mm petri dishes at 37°C. Once solidified, petri dishes were transferred to the temperature-controlled stage on the microscope. As with the tumor cell kinetics experiment, 1.0 ml of 1.0 M mannitol in DMEM was pipetted onto the agarose gel containing tumor tissues, and images of these tissues were taken every minute for 90 min. Image analysis software (Image-Pro Plus®) was used to determine the surface area of tumor sections at each time point. The surface areas were normalized by their initial values at $t = 0$ and then used to calculate the normalized tumor volumes, assuming the volume change to be isotropic. The experiment was repeated three times for each tumor type; and the mean and the standard deviation are reported.

In Vitro Gene Expression

4T1 tumor cells were cultured and harvested as described above. Cells were resuspended in DMEM to a final concentration of 6.25×10^5 cells/ml. 800 μl aliquots of cell suspension were pipetted into Eppendorf tubes and spun at 2000 rpm for 5 min. The supernatant was removed and cells were resuspended in 800 μl DMEM, containing 0, 0.001, 0.01, 0.1, or 1.0 M mannitol. Cells were incubated at 37°C for 20 min. Then, 5 μg pCMVLuc in 50 μl PBS were mixed with cell suspensions, and the mixtures were transferred into electroporation cuvettes with 4-mm gap (BTX, San Diego, CA, USA). Only the cells in the EP+ groups (see the definition below) were subjected to a pulsed electric field consisting of 5 pulses with 200 V/cm in field strength, 20 ms in pulse duration, and 1 s in interval between pulses. After electroporation, 16 μl of cell suspensions were pipetted into wells of a 96-well plate. The volume of solution in each well was brought to 200 μl with DMEM, and the plates were incubated at 37°C for 24 hrs.

In vitro gene expression was quantified with an In Vivo Imaging System (IVIS, Xenogen, Alameda, CA, USA). 5 μl of D-luciferin (28.5 mg/ml) solution was pipetted into each well. Plates were incubated in the IVIS chamber at 37°C for 20 min, and then a grey scale reference image was obtained under low-level illumination. A bioluminescence image was then acquired by integrating photons emitted from the wells for 60 s. The two images were overlaid, and the luciferase expression was displayed as the number of photons emitted per second per square centimeter per steridian (sr). Luciferase expression was quantified as the total photons emitted from the surface of the well per unit time using Living Image Software (Xenogen) (n = 18 for each treatment group).

Hind Leg Tumor Model

Hind leg tumor models were used to quantify effects of mannitol pretreatment on electric field-mediated gene delivery, in terms of reporter gene expression. 4T1 and B16.F10 cells harvested from culture flasks were centrifuged for 2 min at 176 g and re-suspended in PBS to a final concentration of 2×10^7 cells/ml. Female Balb/C and C57BL/6 mice (18–22 g, Charles River, Raleigh, NC, USA) were used as hosts for 4T1 and B16.F10 tumors, respectively. Mice were anesthetized with an i.p. injection of 80 mg ketamine and 10 mg xylazine per kg body weight. Hairs were removed from the left and right hind legs and 50 μl of cell suspension (~1×10^6 cells) were injected subcutaneously on the quadriceps of each leg. Tumors were allowed to grow until they reached 8 – 10 mm in diameter.

Dorsal Skin-Fold Chamber Tumor Model

Tumors grown in dorsal skin-fold chambers (DSCs) were used to quantify the extent of *in vivo* electrophoresis of pDNA in untreated and mannitol pretreated tumors. 4T1 and B16.F10 cells were harvested as described above and re-suspended to a final concentration of 5×10^7 cells/ml in PBS. Fluorescently labeled, electrically neutral, yellow-green latex microspheres (YG-MS) with a diameter of 1.0 μm (Polysciences, Inc., Warrington, PA, USA) were added to the cell suspension, which were used as a tissue marker during image analysis. Female Balb/C and C57BL/6 mice (22–25 g, Charles River, Raleigh, NC, USA) were used as hosts for 4T1 and B16.F10 tumors, respectively. DSCs were implanted in mice anesthetized with an i.p. injection of 80 mg ketamine and 10 mg xylazine per kg body

weight. The procedure has been described in previous studies 13, 15, 31. In brief, 10 μl of cell suspension (~5×10^5 cells) was injected into the fascia layer at the center of the DSC, and then the DSC was sealed with a sterile glass coverslip. 4T1 and B16.F10 tumors were allowed to grow for 5–6 days and 7–10 days, respectively, before they were used in experiments.

Treatment Groups

Solid tumors were divided into four treatment groups. Tumors in the control group, denoted by M−EP−, received reporter gene pDNA infusion with no mannitol or electric field treatment. Tumors in the second group, denoted by M+EP−, received mannitol pretreatment followed by pDNA infusion but no exposure to electric field. Tumors in the third group, denoted by M−EP+, received pDNA infusion and electric field exposure but no mannitol pretreatment. Tumors in the fourth group, denoted by M+EP+, received mannitol pretreatment followed by pDNA infusion and exposure to pulsed electric field.

In Vivo Gene Expression Studies

A plasmid containing the firefly luciferase gene under the cytomegalovirus (CMV) promoter, pCMVLuc, was used in the gene delivery experiments. Mice bearing hind leg tumors were anesthetized as described previously. In the mannitol pretreated groups, i.e., M+EP− and M+EP+, 50 μl of mannitol in PBS (1 M) was infused into the center of the tumors at a constant flow rate of 1.0 μl/s using a 30-gauge needle and a PHD 2000 infusion pump (Harvard Apparatus, Holliston, MA, USA). 20 minutes later, 50 μl PBS containing 5 μg pCMVLuc was infused into the center of the tumor at the same rate. Tumors in the groups without mannitol pretreatment, i.e., M−EP− and M−EP+, received only the pCMVLuc infusion. In the M−EP+ and M+EP+ groups, a pulsed electric field was applied across the tumors using caliper electrodes at 10 s following pCMVLuc administration. The electric field consisted of 10 pulses with 400 V/cm in field strength, 20 ms in duration, and 1 s in interval between pulses, which was generated from an ECM 830 electro square porator (BTX).

Twenty-four hours following electric field application, transgene expression was quantified using the Xenogen IVIS. During the measurement, mice were anesthetized in an induction chamber with 2% Isoflurane and maintained under anesthesia throughout the course of luciferase measurement. Mice were given an i.p. injection of 50 μl aqueous D-luciferin solution (28.6 mg/ml). Twenty minutes later, mice were placed in the Xenogen IVIS and a grey scale reference image was obtained under low-level illumination. Then, a bioluminescence image was acquired by integrating photons emitted from the animals for 30 s. The two images were overlaid, and the luciferase expression was displayed as the number of photons emitted per second per square centimeter per steridian (sr). Luciferase expression in the tumor was later quantified as the total photons emitted from the surface of the tumor per unit time, using Living Image Software (Xenogen).

pDNA Electromobility Studies

Details of the *in vivo* electromobility study have been described previously 13. Briefly, mice were anesthetized once DSC tumors had reached 3–4 mm in diameter and the cover slip was

removed from the DSC. Approximately 2 μl of 1.0 M mannitol in PBS was microinjected into the center of the tumor. Twenty minutes later, approximately 1 μg of rhodamine-labeled plasmid DNA (Rho-pDNA, 5.1 kb, Gene Therapy Systems, San Diego, CA, USA) in 2 μl PBS was injected into the same location. Then, the anesthetized mice were secured on a custom designed microscope stage on a Model 510 confocal microscope (Zeiss) equipped with a 40× objective. Pulsed electric fields were applied using an ECM 830 electro square porator (BTX) via two stainless steel, parallel plate electrodes mounted on the skin side of the DSC. Pulsed electric fields examined in this study consisted of 10 identical square voltage pulses with a magnitude of 100 or 400 V/cm and a duration of 20 or 50 ms. The interval between consecutive pulses was 1 s. Fluorescence images of the Rho-pDNA and YG-MS were acquired both before and immediately following the application of the 10-pulse sequence. A cross-correlation analysis was performed on these images in two different channels. The analysis was performed independently on channel 1 and channel 2 to determine the displacement vectors of $\vec{D}_{pDNA}$ and $\vec{D}_{MA}$, averaged over the field of view, for Rho-pDNA and YG-MS, respectively. These vectors were used to determine the electric field-induced pDNA movement per pulse, d_p, by

$$d_p = \frac{\left| \vec{D}_{pDNA} - \vec{D}_{MA} \right|}{10} \quad (1)$$

Statistical Analysis

Significance of difference in experimental data between two groups was determined with the Mann-Whitney test. All statistical calculations were performed, using Minitab statistical software (Minitab Inc., State College, PA, USA).

Results

Kinetics of Cell Shrinkage In Vitro and Ex Vivo

The tumor cell and tissue volume changes in response to the treatment with 1.0 M mannitol solution were determined with a series of *in vitro* and *ex vivo* experiments, respectively. The results are shown in Figure 1. Twenty minutes following the treatment, the average reductions in volumes of 4T1 and B16.F10 cells were 50 % and 54 %, respectively. Beyond 20 min, tumor cell volumes continued to decrease but at a much slower rate. For example, the average reductions in volumes at 90 min were 40% and 45%, respectively. Unlike individual tumor cells, 4T1 and B16.F10 tumor tissues *ex vivo* did not exhibit a drastic reduction in volumes. They were 93 % and 105 % of their initial volumes, respectively, at 20 min following the exposure to mannitol solution. Based on these results, the time interval between mannitol pretreatment and intratumoral infusion of pDNA *in vivo* was determined to be 20 min.

Effects of Mannitol on Gene Expression In Vitro

A series of *in vitro* cell transfection experiments were performed to determine effects of mannitol pretreatment on transgene expression in cells transfected with pDNA *in vitro*. The gene expression levels, with and without external electric field, are shown in Figure 2. As

expected, transgene expression was the lowest in suspensions not receiving pulsed electric field treatment, which included the two control groups (nC and pC) and the group with 1.0 M mannitol but not subjected to an external electric field. Statistically, there were no differences in transgene expressions among these groups ($P > 0.05$). The application of a pulsed electric field enhanced transgene expression in cells suspended in the agarose gel ($P < 0.05$), containing mannitol at concentrations of 0, 0.001, 0.01, and 0.1 M, respectively. The expression levels between these groups were not significantly different ($P > 0.05$), indicating that mannitol pretreatment had little effects on electric field-mediated gene delivery *in vitro*. However, the transgene expression in cell suspensions containing mannitol at 1.0 M was not significantly enhanced by pulsed electric field ($P > 0.05$), indicating that mannitol at this concentration had antagonist effects on electric field-mediated transfection.

Effects of Mannitol Treatment on Gene Expression In Vivo

Effects of mannitol pretreatment on electric field-mediated gene delivery *in vivo* were quantified via reporter gene expression. The average gene expressions in each treatment group for both tumors are shown in Figure 3. As expected, electric field treatment alone resulted in a significant increase in gene expression in both 4T1 and B16.F10 tumors ($P < 0.05$). Mannitol pretreatment alone did not alter reporter gene expression in either tumor type ($P > 0.05$). Tumors in the M+EP+ group had a significantly higher level of gene expression than those in any of the other treatment groups, including the M−EP+ group, for both tumors ($P < 0.05$). These data indicated that mannitol pretreatment prior to the application of pulsed electric field could enhance electric field-mediated gene delivery in solid tumors.

Effects of Mannitol Pre-treatment on pDNA Electrophoresis In Vivo

To understand mechanisms of the enhancement in gene delivery induced by mannitol pretreatment, pDNA electromobility was measured *in vivo* in 4T1 and B16.F10 tumors grown in DSC. The average distances of electric field-induced transport in untreated and mannitol-pretreated tumors are shown in Figure 4. These data indicated that the mannitol pretreatment did not result in a significant increase in pDNA electrophoresis in either 4T1 or B16.F10 tumor ($P > 0.05$).

Discussion

Effects of mannitol pretreatment of tumor tissues on electric field-mediated gene delivery were explored in this study. It was observed that the hyperosmotic solution of mannitol reduced the volume of cells and increased interstitial volume fraction in tumor tissues. In addition, the pretreatment significantly improved electric field-mediated gene delivery but did not change pDNA electromobility in tumor tissues *in vivo*.

The exposure of tumor cells to the hyperosmotic mannitol solution significantly reduced the cell volume, due to the induced osmotic pressure gradient across the cell membrane. However, the rebound swelling, a phenomenon reported in the literature for astroglial cells due to an active mannitol uptake 32, was not observed in our experiments for 4T1 and B16.F10 cells. The kinetic studies also showed that neither 4T1 nor B16.F10 tissues

underwent a significant reduction in volume following *ex vivo* exposure to mannitol solution (see Figure 1b). The volume fractions of cells in 4T1 and B16.F10 tumors prior to the mannitol treatment were 18% and 33%, respectively, which were measured in a previous study 21. The volume fraction of microvessels in tumor tissues *ex vivo* was negligible due to the vessel collapse after the tumors were removed from animals. Therefore, the estimated volume fractions of the interstitial space were 82% and 67% in 4T1 and B16.F10 tumors, respectively. The combination of these data and those shown in Figure 1 suggested that the pretreatment of tumors with the hyperosmotic mannitol solution increased the volume fractions of the interstitial space from 82% to 90% in 4T1 tumors and from 67% to 83% in B16.F10 tumors. Changes in the volume fraction of the interstitial space were expected to be less significant and more heterogeneous *in vivo* than *ex vivo* because of the interstitial spreading of mannitol as well as clearance of this molecule through microvessels 33. Therefore, the *ex vivo* data reported were the upper bounds of the increase in interstitial volume fraction of the same tumors *in vivo*.

The results shown in Figure 2 demonstrated that transgene expression was increased significantly following the exposure of tumor cells to pulsed electric field, but no additional change in transgene expression was observed when the cells were pretreated with mannitol solutions with a wide range of concentrations. Furthermore, the transgene expression was significantly reduced ($P < 0.05$) when the mannitol concentration was increase to 1.0 M. The reduction might be caused by three potential mechanisms. First, the hyperosmotic solution of mannitol might be toxic to cells although the cell damage was not obvious in terms of the cell morphology under a light microscope. Additionally, cell counts appeared to be consistent with those observed in other experimental groups. Second, the external electric field induced transmembrane potential is proportional to the cell radius 16, 34. In order to reach the threshold value of the transmembrane potential (~ 1 V) for initiating electroporation of cell membrane, the smaller cells require a stronger applied electric field. Thus, it was possible that membranes of smaller cells, due to the exposure to 1.0 M mannitol solution, were not permeabilized by the applied electric field. Third, the transgene expression could be inhibited directly by the hyperosmotic stresses at both transcriptional and translational levels 35–38.

Bioluminescence technique was used to investigate effects of mannitol pretreatment on electric field-mediated gene delivery *in vivo*. Without mannitol pretreatment, a pulsed electric field (400 V/cm, 20 ms, 1 s intervals, n = 10) alone could significantly increase the reporter gene expression in both 4T1 and B16.F10 hind leg tumors. The data were consistent with those in previous studies 9, 13, 14, 39–42. The pretreatment of tumors with 1.0 M mannitol solution further increased the reporter gene expression in both 4T1 and B16.F10 tumors (see Figure 3). The additional increase was likely to be due to an improvement in interstitial transport of pDNA rather than an increase in cell permeability or changes in intracellular microenvironment since the enhancement was not observed in the *in vitro* experiment (see Figure 2). Here, it is important to note that the mannitol concentration in tumors *in vivo* was less than 1 M when comparing the data to those shown in Figure 2. As discussed above, the reduction in the mannitol concentration was due to interstitial spreading and clearance of this molecule through microvessels.

To investigate how mannitol pretreatment affected the interstitial transport of pDNA, DSC tumor models were used to directly quantify pDNA electromobility *in vivo* following mannitol pretreatment, and the results were compared with those in tumors that did not receive mannitol pretreatment. Despite the enhanced gene expression levels, the mannitol pretreatment had minimal effects on pDNA electromobility *in vivo* (see Figure 4). The unexpected result might be explained by the following two scenarios.

One was that the hind leg and DSC tumor models were significantly different from each other since tumor pathophysiology depends on the site of tumor implantation 19. Additionally, the dose of mannitol administered into the hind leg tumors was 25-fold greater than that into the DSC tumors because the latter was much smaller in size than the former. The dose difference might affect the extracellular concentration of mannitol, which was the driving force behind the reduction in the cell volume fraction or the increase in the interstitial volume fraction. However, it was unlikely for mannitol pretreatment to have no effects on the volume fraction of cells in the DSC tumors.

The second scenario is shown schematically in Figure 5. The reduction in cell volume, induced by hyperosmotic mannitol solution, could increase the spacing between cells and between cell and extracellular matrix (ECM). These changes in tissue structures would increase (i) the pore size in the interstitial space, (ii) the thickness of interstitial fluid layer on the surface of tumor cells, and (iii) the surface area of cells exposed to interstitial fluid. As a result, they improved pDNA distribution in tumors prior to the application of pulsed electric field and allowed more pDNA molecules to be in direct contact with the cell membrane instead of being trapped by ECM, which is one of the main obstacles for pDNA delivery in tumors as demonstrated in a previous study 13. This scenario could explain why mannitol pretreatment caused an increase in gene delivery but no enhancement in pDNA electrophoresis over a few micron-distance, during which pDNA would bind inevitably to collagen fibers. Similar results have been observed in a previous study, in which collagen remodeling, induced by a recombinant human relaxin, significantly increased transgene expression in 4T1 tumors when they grew subcutaneously in the hind leg. However, the relaxin treatment could minimally alter pDNA electromobility when the tumors were implanted in the DSC 13.

In summary, mannitol pretreatment was an effective method for enhancing electric field-mediated gene delivery in solid tumors. The enhancement was likely to be due to the pretreatment-induced increase in interstitial fluid space, which could improve both the distribution volume of pDNA and the local concentration of unbound pDNA in pericellular regions during intratumoral infusion. The improvement would in turn increase the number of transfected cells and the average number of pDNA molecules per cell during electroporation. The mechanisms of mannitol pretreatment-mediated enhancement in gene delivery can also be applied to improving drug delivery in solid tumors.

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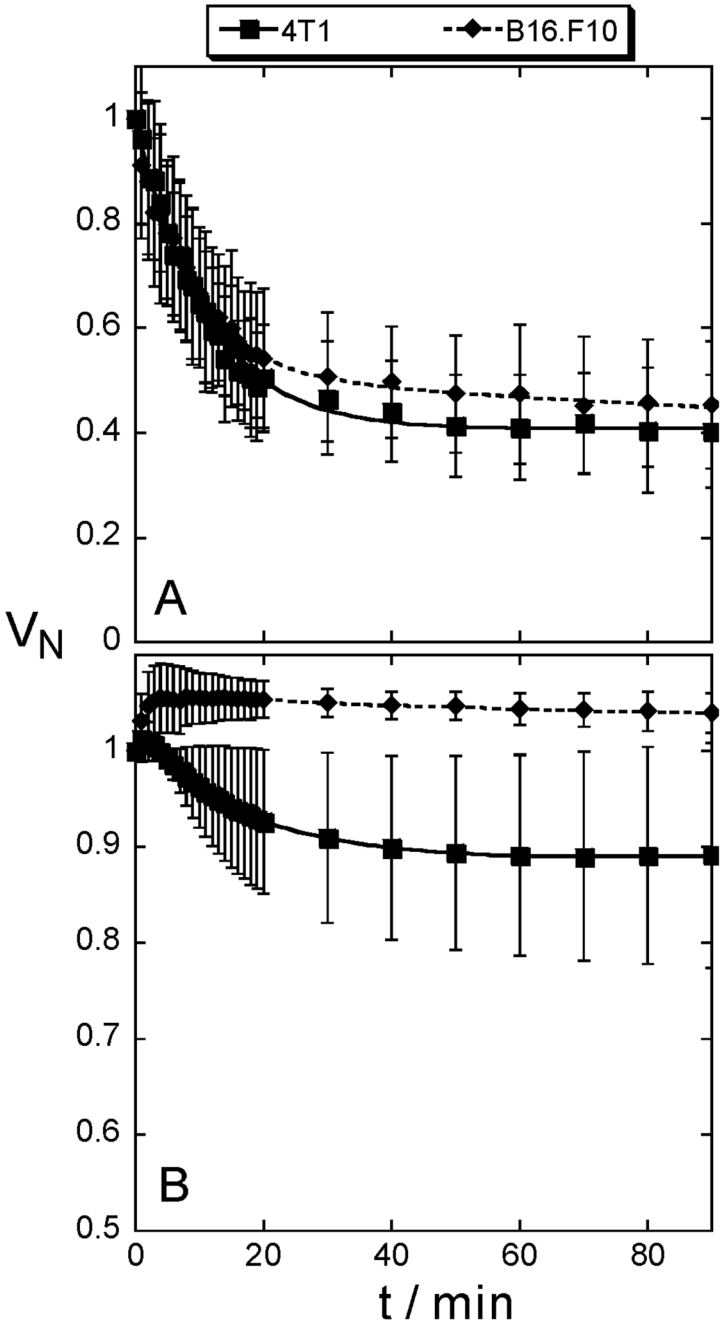

Figure 1.

Changes in cell volume (A) and excised tumor tissue volume (B) in response to treatment with hyperosmotic solution of mannitol. V_N represents the cell or tissue volume normalized by its pre-treatment volume at t = 0. The symbols in the plots represent the mean value and the error bars represent the standard deviation. n = 48 for cell samples and n = 3 for tissue samples.

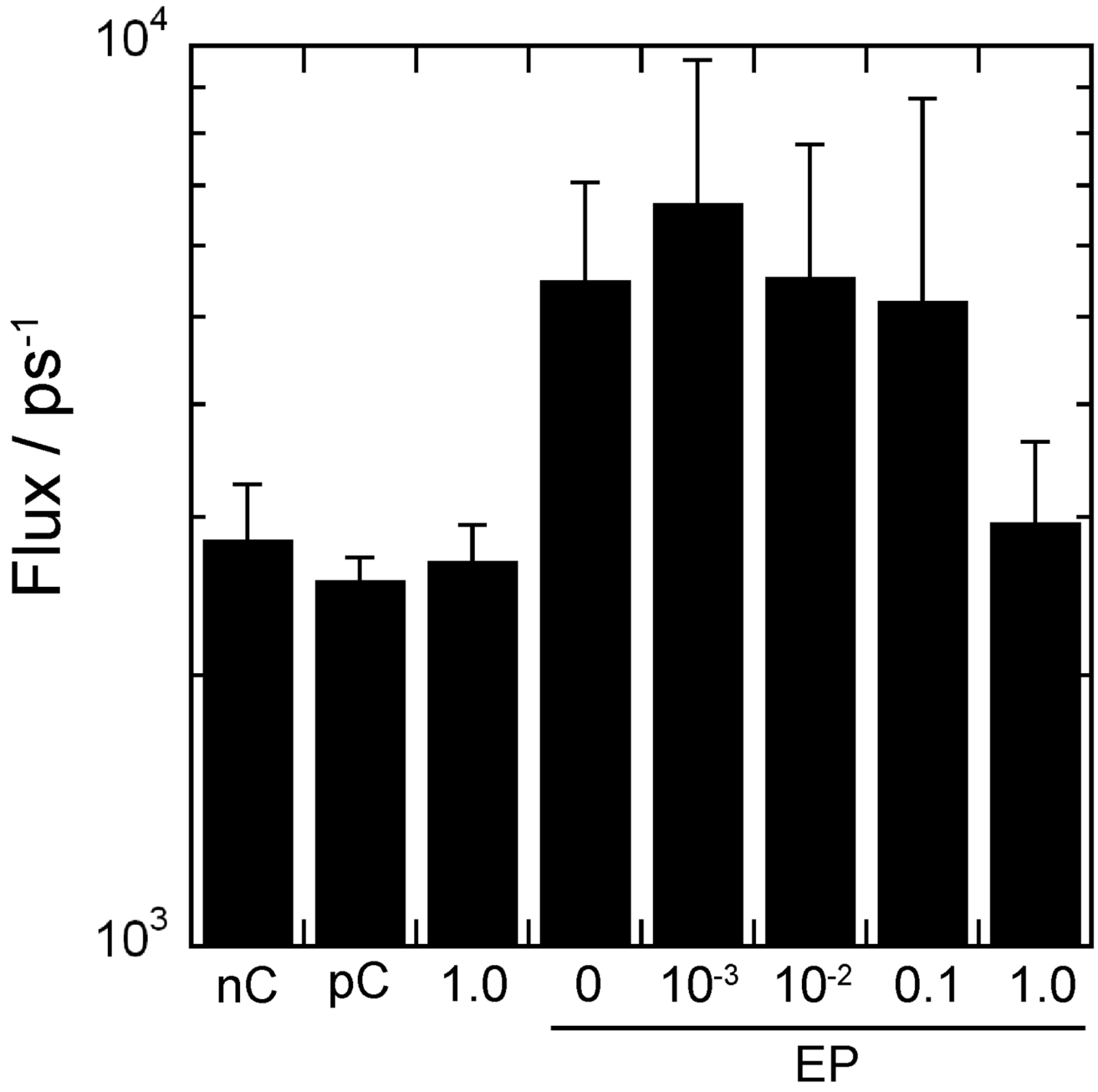

Figure 2.

In vitro luciferase expression in 4T1 cell suspensions containing mannitol with a range of concentrations. The molar concentration of mannitol in a given treatment group is indicated by the numerical value on the x-axis. nC and nP denote the control groups with and without plasmid, respectively. Cells in all electroporation (EP) groups were treated with a pulsed electric field consisting of 5 pulses with 200 V/cm in field strength, 20 ms in duration, and 1 s in interval between pulses. Bars show the mean value and error bars represent the standard deviation. n = 18 for each group.

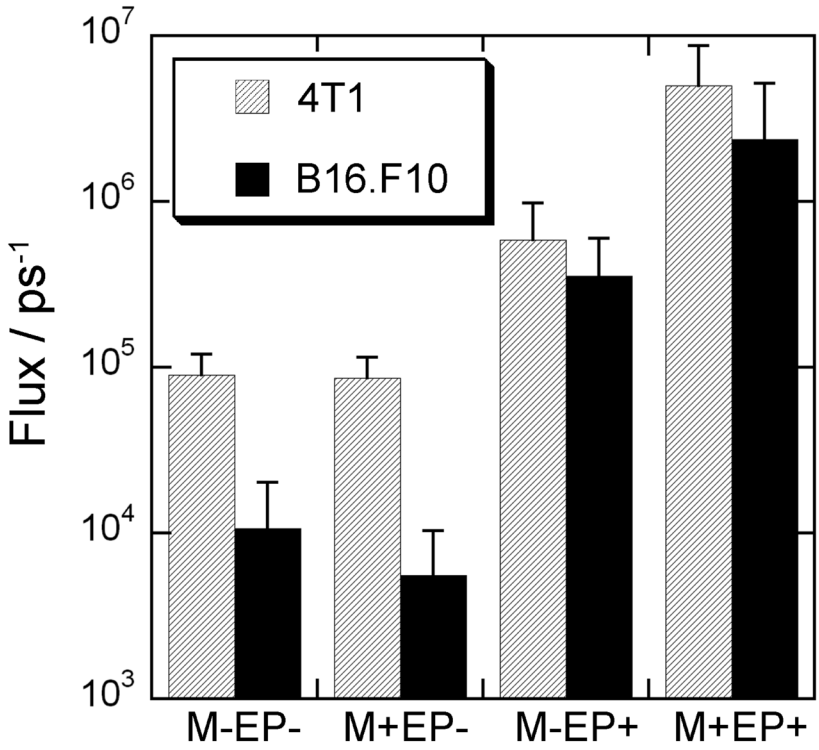

Figure 3.

In vivo gene expression in hind leg 4T1 and B16.F10 tumors. The treatment groups were as follows: M−EP−, tumors received pDNA infusion only; M+EP−, tumors received mannitol pretreatment and pDNA infusion; M−EP+, tumors received pDNA infusion and exposed to pulsed electric field; M+EP+, tumors received mannitol pretreatment and pDNA infusion followed by exposure to pulsed electric field. Bars show the mean of total photons emitted per second across the surface of the tumors and the error bars represent the standard deviation from the mean. n = 15 for M−EP−, n = 5 for M−EP+, M+EP− and M+EP+. For each tumor model, there were no differences in the data between M−EP− and M+EP− groups ($P > 0.05$); the data in M−EP+ and M+EP+ groups were higher than those in M−EP− and M+EP− groups for each tumor model ($P < 0.05$); and cells in the M+EP+ group had a significantly higher level of gene expression than those in the M−EP+ group ($P < 0.05$).

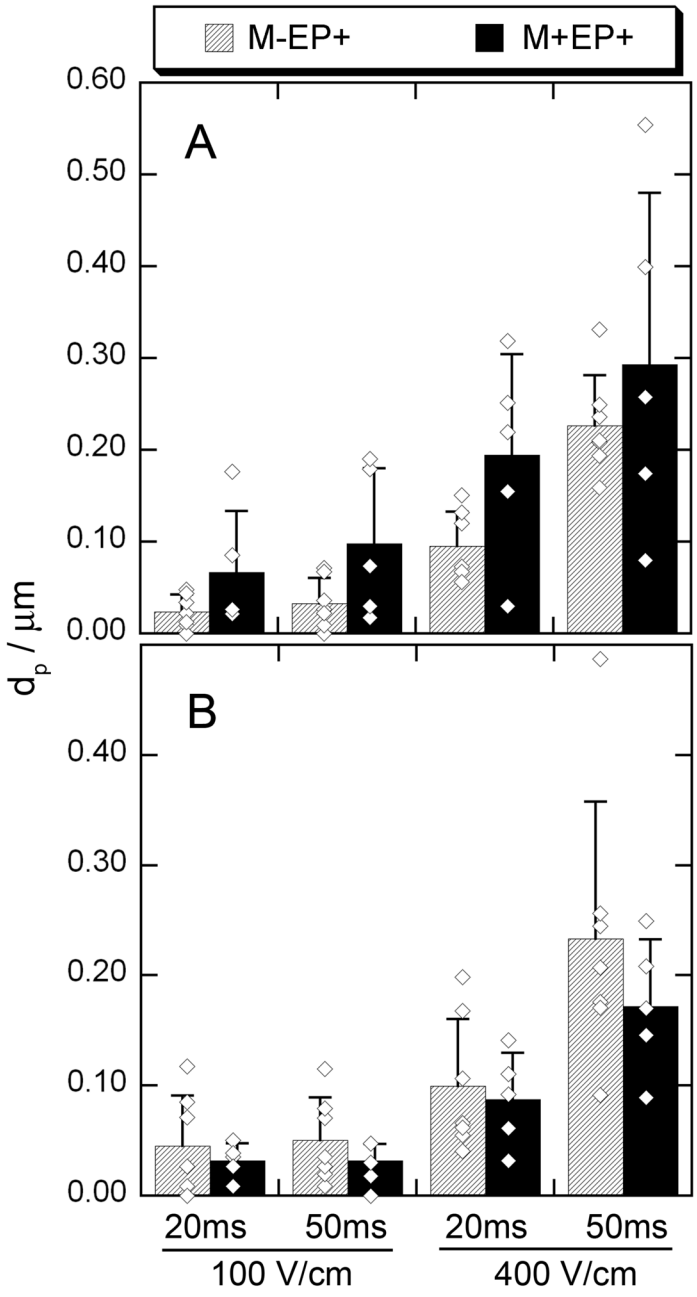

Figure 4.

Net distance of pDNA electrophoretic movement per pulse in untreated (grey) and mannitol pretreated (black) 4T1 (A) and B16.F10 (B) tumors grown in the DSCs. The applied electric field consisted of 10 square wave pulses with 100 or 400 V/cm in strength, 20 or 50 ms in duration, and 1 s in interval between pulses. The symbols represent individual data points, bars represent the mean value, and error bars represent the standard deviation from the mean. n = 7 for untreated and n = 5 for mannitol pretreated tumors.

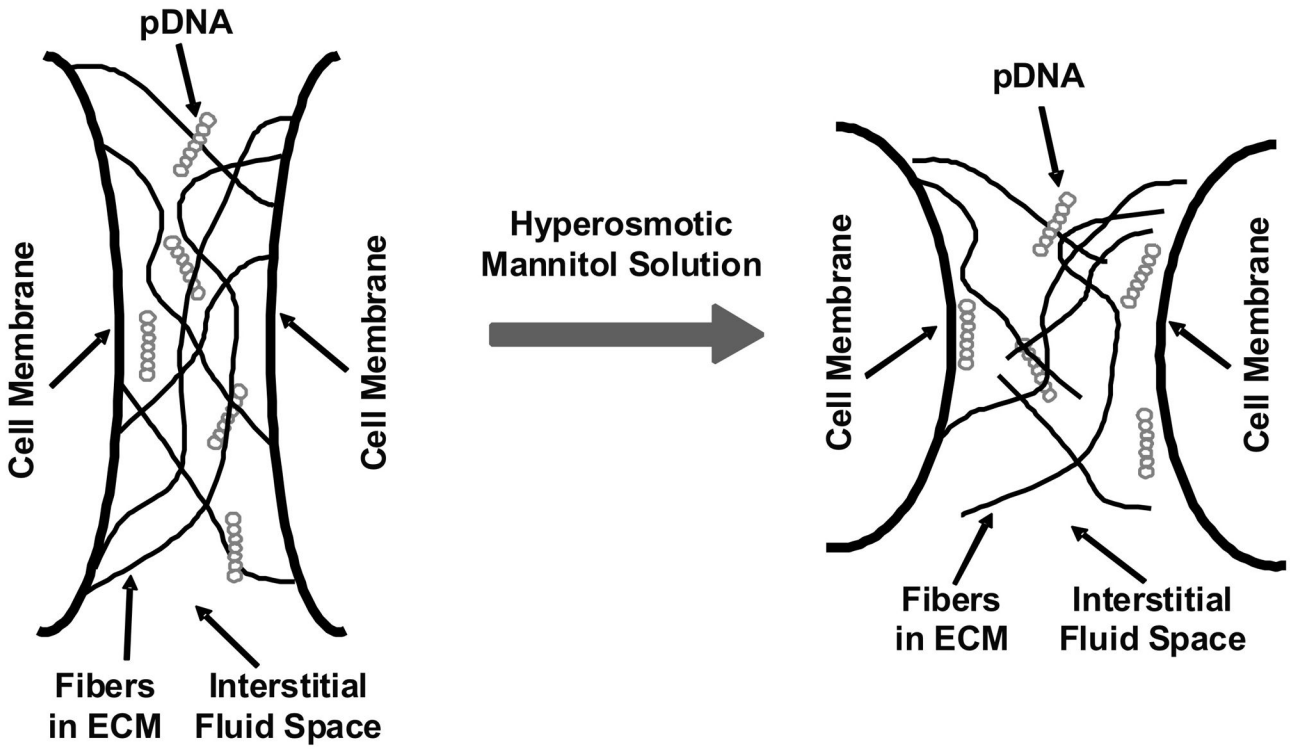

Figure 5.

A potential mechanism of mannitol pretreatment-mediated enhancement in pDNA delivery into tumor cells *in vivo*. The pretreatment reduced the size of cells and subsequently caused remodeling of extracellular matrix (ECM). These changes would increase the size of pores in ECM and the interstitial fluid space in the vicinity of cell membrane. The increase in the pore size would reduce interstitial resistance to convective transport of pDNA during the intratumoral infusion; and the increase in the interstitial fluid space would increase the number of pDNA molecules that were non-trapped by collagen fibers or directly in contact with the cell membrane. As a result, pDNA could spread to a larger volume in solid tumors during the infusion and more pDNA molecules could move across the permeabilized regions in the plasma membrane during electroporation. All of these would increase pDNA delivery into tumor cells *in vivo*. On the other hand, the changes in cell volume and ECM structure mentioned above would not necessarily enhance pDNA electrophoresis over a few micron-distance because of the multivalent binding between pDNA and collagen fibers.