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Influence of Plasmid Concentration on DNA Electrotransfer In Vitro Using High-Voltage and Low-Voltage Pulses

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Abstract DNA electrotransfer in vivo for gene therapy is a promising method. For further clinical developments, the efficiency of the method should be increased. It has been shown previously that high efficiency of gene electrotransfer in vivo can be achieved using high-voltage (HV) and low-voltage (LV) pulses. In this study we evaluated whether HV and LV pulses could be optimized in vitro for efficient DNA electrotransfer. Experiments were performed using Chinese hamster ovary (CHO) cells. To evaluate the efficiency of DNA electrotransfer, two different plasmids coding for GFP and luciferase were used. For DNA electrotransfer experiments 50 µl of CHO cell suspension containing 100, 10 or 1 µg/ml of the plasmid were placed between plate electrodes and subjected to various combinations of HV and LV pulses. The results showed that at 100 µg/ml plasmid concentration LV pulse delivered after HV pulse increased neither the percentage of transfected cells nor the total transfection efficiency (luciferase activity). The contribution of the LV pulse was evident only at reduced concentration (10 and 1 µg/ml) of the plasmid. In comparison to HV (1,200 V/cm, 100 µs) pulse, addition of LV (100 V/cm, 100 ms) pulse increased transfection efficiency severalfold at 10 µg/ml and fivefold at 1 µg/ml. At 10 µg/ml concentration of plasmid, application of four LV pulses after HV pulse increased transfection efficiency by almost 10-fold. Thus, these results show that contribution of electrophoretic forces to DNA electrotransfer can be investigated in vitro using HV and LV pulses.

Keywords Gene transfer · DNA electrotransfer · DNA electrophoresis · Gene expression · Electroporation · Electropermeabilization

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Introduction

Delivery of genetic material for gene therapy can be achieved using viral and nonviral methods (Bouard et al. 2009; Seow and Wood 2009; Kawakami et al. 2008). Viral vectors are considered to be the most efficient method for gene transfer, but often they are associated with host inflammatory and immune responses (Frank et al. 2009). Therefore, alternative nonviral chemical and physical methods of gene delivery are under intense investigation. Among the physical methods the most promising is electroporation, a method based on the application of electric pulses to cells (Mir et al. 1999; Mir 2009). In comparison to widely accepted viral and chemical vectors, DNA electrotransfer has proved a simple, cheap, nontoxic and safe method for transfer of foreign genes into cells and tissues. The method involves injection of the plasmid into target tissue and application of electric pulses at the injected site (Mir et al. 1999; Heller and Heller 2006; Cemazar et al. 2006a, b). However, so far the efficiency of gene transfer using electroporation (electrotransfer) is insufficient, and this remains the main limitation for further development of the method for clinical trials. Optimization

of the method is difficult since the mechanisms as well as optimal conditions for an effective gene electrotransfer are still largely unknown (Golzio et al. 2002; Cemazar et al. 2006a, b; Escoffre et al. 2009).

The effectiveness of gene electrotransfer in vivo can be increased using two types of electric pulses (Bureau et al. 2000; Satkauskas et al. 2002). It is suggested that one short-duration high-voltage (HV) pulse is mainly responsible for cell electroporation, while a second longer-duration low-voltage (LV) pulse electrophoretically moves DNA into the cell (Klenchin et al. 1991; Sukharev et al. 1992). It was demonstrated in vivo that LV pulse governs DNA electrotransfer efficiency, presumably by exerting electrophoretic forces that can interact with DNA and facilitate its translocation across the membrane into porated cells (Satkauskas et al. 2002).

While electrophoretic forces after cell electroporation have been shown to play a significant role in DNA delivery into cells in vivo (Satkauskas et al. 2002, 2005), for in vitro conditions such forces have been found to be unnecessary (Cepurniene and Satkauskas, 2008). Indeed, using GFP coding plasmid at a concentration of 100 μg/ml, the percentage of transfected cells using HV + LV pulse combinations did not depend on LV pulses. In that case the number of green fluorescent protein (GFP)-positive cells mainly depended on the parameters of the HV pulse (Cepurniene and Satkauskas 2008). On the other hand, several previous studies have clearly implicated electrophoretic forces in DNA electrotransfer (Klenchin et al. 1991; Sukharev et al. 1992; Neumann et al. 1996). In addition, Kanduser et al. (2009) recently showed the importance of LV pulses in vitro on plated cells using suboptimal plasmid concentration. Although the authors demonstrated that LV pulse following four HV pulses increased significantly the number of transfected cells, they did not show how transfection efficiency depended on LV pulse parameters (Kanduser et al. 2009). In our study using HV and various LV pulses we aimed to quantitatively investigate the contribution of the LV pulse to transfection efficiency dependent on plasmid concentration.

Materials and Methods

Cells

Chinese hamster ovary (CHO) cells were grown as a monolayer culture in complete DMEM at 37°C in humidified 5% CO_2 atmosphere in the incubator. After trypsinization, cells were suspended in low-conductivity electroporation medium (0.25 M glucose, 1 mM $MgCl_2$, 10 mM Na_2HPO_4 [pH 7.4]).

Plasmids

GFP coding plasmid pMAX-GFP (Amaxa, Cologne, Germany) of 3,485 bp and luciferase coding plasmid pGL4.13 (Promega, Madison, WI) of 4,641 bp were used in the experiments. GFP coding plasmid was used to evaluate the percentage of transfected cells, while luciferase coding plasmid was used to evaluate total transfection efficiency (luciferase activity). Plasmids were amplified in *Escherichia coli* and purified using the EndoFree Plasmid MaxiPrep kit (Qiagen, Chatsworth, CA) following the manufacturer's instructions. The concentration and quality of the plasmid preparations were controlled by spectrophotometry and gel electrophoresis.

Electroporator

To generate electric pulses, we used an electroporator developed in our laboratory in collaboration with Kaunas University of Technology. The electroporator enables one to generate various combinations of HV and LV pulses. For the delivery of electric pulses stainless-steel plate electrodes were used. The distance between the plates of the electrodes was 2 mm.

DNA Electrotransfer

Two million CHO cells were suspended in 1 ml of low conductivity electroporation medium. In each group 5×10^4 cells (45 μl) were taken from the cell suspension. Plasmid (5 μl) was put in 45 μl of the cell suspension. Three final plasmid concentrations were used: 100, 10 and 1 μg/ml. All experiments, if not stated otherwise, were performed using one HV and/or one LV. The parameters of the HV pulse, such as pulse strength of 1,200 V/cm and pulse duration of 100 μs, were kept constant in all experiments, while pulse strength and pulse duration of the LV pulse, depending on the experiment, varied from 0 to 100 V/cm and from 60 to 100 ms, respectively. The delay between HV and LV pulses was 1 s. After cell electroporation with different combinations of HV and LV pulses, cells were kept for 10 min at room temperature. For the experiments with luciferase coding plasmid, 10^4 treated cells were plated in the wells of microplates (Plastibrand; Brand, Wertheim, Germany) and allowed to grow in cell culture medium for 24 h. Luciferase protein activity was measured with the ONE-Glo™ Luciferase Assay System (Promega) using a luminometer (Tecan GENios Pro; MTX Lab Systems, Vienna, VA) and is shown in figures as relative light units (RLUs). For experiments with GFP coding plasmid, 10^5 cells were plated in 35-mm Petri dishes and allowed to grow in cell culture medium for 24 h. The cells then were counted under a fluorescent microscope (BA

400; Motic, Wetzlar, Germany) in six randomly taken images. Transfection efficiency was determined as a ratio between GFP-positive cells in the experimental sample and the total number of cells in the control sample. Cell viability was determined as a ratio of the total number of cells in the experimental sample and the total number of cells in the control sample.

Statistical Analysis

Each experimental point was obtained from at least three independent experiments, and results are represented as mean ± standard error of the mean. The statistical significance of differences between the groups was evaluated by two-sided, unpaired Student's t test (NS, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$).

Results and Discussion

In order to reveal the importance of electrophoretic force for DNA electrotransfer into CHO cells using HV + LV pulse combinations, we set up our experiments to evaluate the efficiency of DNA electrotransfer dependent on LV pulse strength. One 100-ms duration LV pulse at pulse strength of 0–100 V/cm was delivered after one 100-μs duration HV pulse at pulse strength of 1,200 V/cm. In order to evaluate the percentage of transfected cells (GFP-positive cells), experiments were performed using GFP coding plasmid at 100 μg/ml (Fig. 1). A high concentration

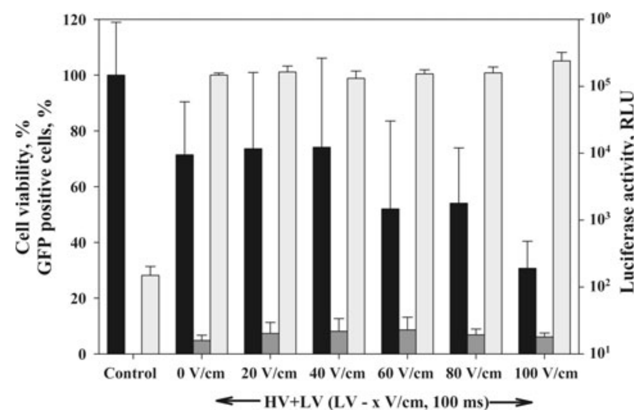

Fig. 1 Cell viability (*black bars*), rate of GFP-positive cells (*dark gray bars*) and luciferase activity (*gray bars*) after cell treatment with various HV + LV pulse combinations dependent on LV pulse strength. All experiments were performed using one HV of 1,200 V/cm pulse strength and 100 μs pulse duration and one LV of various pulse strengths (0, 20, 40, 60, 80 or 100 V/cm) and 100 ms pulse duration. The delay between HV and LV pulses was 1 s. Concentration of plasmid were 100 μg/ml. Variations between the number of GFP-positive cells and luciferase activity comparing HV + LV groups were not significant

of the plasmid itself did not induce any significant cell toxicity in the absence or presence of cell treatment with electric pulses (data not shown). As shown in Fig. 1, a stepwise increase of LV pulse strength resulted in a gradual decrease of cell viability. Irrespectively, the percentage of GFP-positive cells remained nearly constant and leveled near 10%.

A similar result was recently obtained by Kanduser et al. (2009). The authors suggested that at sufficiently high (optimal) concentration of the plasmid, when enough DNA molecules are present in close proximity of the cells, HV pulse alone brings enough plasmid to electroporated cells. Under these conditions, one or several LV pulses do not increase the percentage of GFP-positive cells (Kanduser et al. 2009). However it remained unclear whether the LV pulse could deliver more plasmid in the same percentage of cells as the HV pulse alone. To answer this question, we performed DNA electrotransfer experiments with HV and LV pulses using luciferase coding plasmid at 100 μg/ml. Quantification of luciferase activity in transfected cells showed no difference between HV and all of the HV + LV groups (Fig. 1). Therefore, it could be concluded from Fig. 1 that LV pulse delivered 1 s after HV pulse had no effect on either the increase of the number of transfected cells (percentage of GFP-positive cells) or the total transfection efficiency (luciferase activity).

Kanduser et al. (2009) proposed that the importance of the LV pulses for DNA electrotransfer in vitro can be masked by the excess of plasmid in cell suspension. They showed that at suboptimal plasmid concentration LV pulse significantly increased transfection efficiency in comparison to HV pulse alone. Therefore, in further studies we tested the importance of LV pulse by performing experiments with 10- and 100-fold reduced concentration of plasmid (10 and 1 μg/ml). As at the low concentration of GFP coding plasmid due to low fluorescence signal it was not possible to reliably evaluate GFP-positive cells, for these types of experiments we used luciferase coding plasmid. Experiments were performed using one 100-μs duration HV pulse at 1,200 V/cm and one LV pulse at 100 V/cm. Several LV pulse durations (60, 80 and 100 ms) were tested (Fig. 2).

The results showed that the importance of the LV pulse was more evident for reduced concentrations of the plasmid. At these concentrations, luciferase activity increased with the increase of the duration of the LV pulse from 60 to 100 ms. At the lowest plasmid concentration tested (1 μg/ml) and LV pulse duration of 80 and 100 ms, luciferase activity in the HV + LV group was significantly larger than that in the HV group (Fig. 2). To evaluate the contribution of the LV pulse for DNA electrotransfer, we estimated the increase of luciferase activity in the HV + LV group in comparison to the HV pulse (Table 1).

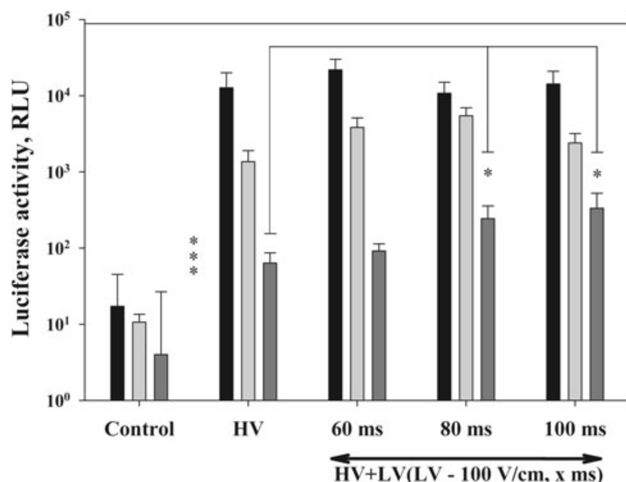

Fig. 2 Luciferase activity after DNA electrotransfer using HV and LV pulses dependent on luciferase coding plasmid concentrations: 100 μg/ml (*black bars*), 10 μg/ml (*gray bars*) and 1 μg/ml (*dark gray bars*). All experiments were performed using one HV of 1,200 V/cm pulse strength and 100 μs pulse duration and one LV of 100 V/cm pulse strength with various pulse durations (60, 80 or 100 ms). The delay between HV and LV pulses was 1 s. * $P < 0.05$, *** $P < 0.005$. Luciferase activity in the HV + LV groups at 100 and 10 μg/plasmid concentration was not significantly different from that of HV groups

Table 1 Contribution of LV pulse to the DNA electrotransfer into CHO cells in vitro, represented as a ratio of luciferase activity at HV + LV and HV pulse conditions dependent on plasmid concentration

Ratio of luciferase activity	100 μg/ml	10 μg/ml	1 μg/ml
$\frac{\text{HV + LV (100 V/cm, 60 ms)}}{\text{HV}}$	1.46	2.81	1.47
$\frac{\text{HV + LV (100 V/cm, 80 ms)}}{\text{HV}}$	0.85	4.01	3.82
$\frac{\text{HV + LV (100 V/cm, 100 ms)}}{\text{HV}}$	1.12	1.76	5.23

HV pulse strength was 1,200 V/cm, duration 100 μs. Parameters of the LV pulse are shown in parentheses. The delay between HV and LV pulses was 1 s

It is evident from Table 1 that the ratio between the HV + LV and HV groups increased with the increase in LV pulse duration and decrease in plasmid concentration. The highest increase in our experiments was 5.23 at 100 ms LV pulse duration and 1 μg/ml plasmid concentration.

In order to see whether the conditions of the LV pulses at reduced plasmid concentration could be optimized in the HV + LV pulse combinations, in subsequent experiments we increased the number of LV pulses. The experiments were performed using one 100-μs duration HV pulse at 1,200 V/cm and one, two or four 100-ms duration LV pulses at 100 V/cm (Fig. 3). Since at the lowest plasmid concentration (1 μg/ml) luciferase activity in transfected cells was variable and only 10 times higher than in control (see Fig. 2), to get more statistically reliably data the experiments were performed at an intermediate 10 μg/ml concentration of luciferase coding plasmid.

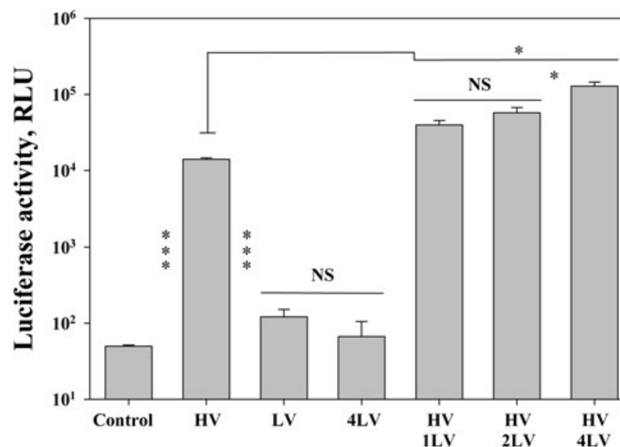

Fig. 3 Luciferase activity after DNA electrotransfer using various HV and LV pulse combinations. All experiments were performed with one HV of 1,200 V/cm, 100 μs, and one, two or four LVs of 100 V/cm, 100 ms. The delay between HV and LV pulses was 1 s. Concentration of plasmid in all groups was 10 μg/ml. * $P < 0.05$, *** $P < 0.005$; *NS* not significant

As expected, an increase in the number of LV pulses from one to four resulted in increased expression of luciferase. As in Fig. 2 and Table 1, in comparison to HV pulse, HV + LV pulses resulted in a 2.8-fold increase in luciferase activity. Notably, addition of four LV pulses increased luciferase activity by almost 10-fold (Fig. 3). One LV or four LV pulses applied without HV pulse had no effect on luciferase expression, and the signal was similar to the control (cells and plasmid). The result that four LV pulses following an HV increased luciferase activity to a higher extent than one LV pulse can be explained by increased electrophoretic force exerted by four LV pulses. Then, more plasmid molecules could be transferred from the bulk and trapped into membranes of electroporated cells. On the other hand, since application of four LV pulses following HV resulted in a decrease by 10% of cell viability in comparison to one LV, four LV pulses could also increase the level of cell permeabilization. This would result in an increased number of transfected cells and/or the number of transferred plasmids to electroporated cells. These questions remain to be clarified in forthcoming studies.

In conclusion, our results clearly show the importance of the LV pulse for DNA electrotransfer using HV + LV pulses. In agreement with Kanduser et al. (2009), we demonstrate that an increase in transfection efficiency by LV pulses is evident only at reduced plasmid concentration. We show that this increase is more pronounced with more reduced concentration of plasmid. Moreover, the results showing that at reduced plasmid concentration

transfection efficiency is dependent on LV pulse duration and LV pulse number indicate that the importance of the electrophoretic pulse as well as the mechanisms of DNA electrotransfer can be investigated in vitro using HV and LV pulses. Overall, our study reinforces the previous hypothesis that LV pulses act on DNA and presumably bring DNA from the bulk of suspension into close contact with the electroporated membranes of cells. Together with results of previous studies this study allow us to hypothesize that LV pulses at reduced plasmid concentration not only increase the percentage of transfected cells but also increase the quantity of plasmid that is transferred into the cells and is expressed.

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