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The Role of Electrophoresis in Gene Electrotransfer

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Abstract Gene electrotransfer is an established method for gene delivery which uses high-voltage pulses to increase the permeability of a cell membrane and enables transfer of genes. Poor plasmid mobility in tissues is one of the major barriers for the successful use of gene electrotransfer in gene therapy. Therefore, we analyzed the effect of electrophoresis on increasing gene electrotransfer efficiency using different combinations of high-voltage (HV) and low-voltage (LV) pulses in vitro on CHO cells. We designed a special prototype of electroporator, which enabled us to use only HV pulses or combinations of LV + HV and HV + LV pulses. We used optimal plasmid concentrations used in in vitro conditions as well as lower suboptimal concentrations in order to mimic in vivo conditions. Only for the lowest plasmid concentration did the electrophoretic force of the LV pulse added to the HV pulse increase the transfection efficiency compared to using only HV. The effect of the LV pulse was more pronounced for HV + LV, while for the reversed sequence, LV + HV, there was only a minor effect of the LV pulse. For the highest plasmid concentrations no added effect of LV pulses were observed. Our results suggest that there are different contributing effects of LV pulses: electrophoretically increased contact of DNA with the membrane and increased insertion of DNA into permeabilized cell membrane and/or translocation due to electrophoretic force, which appears to be the dominant effect.

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Introduction

Gene electrotransfer of cells was first achieved over 25 years ago (Neumann et al. 1982). It combines addition of plasmid DNA and local application of electric pulses (electroporation), which increase the permeability of the membrane (Pavlin et al. 2008) and consequently enable delivery of DNA into the cell and expression of a gene. Gene electrotransfer is already an established method for gene transfer in vitro, and it is currently (Escoffre et al. 2009) being extensively studied on animal models in vivo, with the first clinical trials being reported (Prud'homme et al. 2006; Daud et al. 2008). In comparison to viral gene therapy, gene electrotransfer represents a safer method which is not hampered in terms of immunogenicity and pathogenicity (Ferber 2001). Recent studies showed that gene electrotransfer is a promising method for cancer gene therapy, DNA vaccination, autoimmune and inflammatory diseases as well as other illnesses (Tang et al. 1992; Li and Huang 2000; Fewell et al. 2005).

Up to now several mechanisms have been proposed for electric field-mediated gene transfer. The first hypothesis suggested that the electric pulses create pores in the cell membrane and that these membrane pores consequently enable free diffusion of DNA molecules through the membrane (Neumann et al. 1982) due to the concentration gradient, similar to the diffusion of small molecules. However, further studies showed that delivery of DNA molecules across the cell membrane is a

much more complex process which cannot be explained by simple diffusion through membrane pores (Wolf et al. 1994). By direct visualization of DNA complexes on the cell membrane it was demonstrated that one of the crucial steps is the interaction of DNA molecules with the cell membrane (Golzio et al. 2002; Reberšek et al. 2007). Currently, several steps that are involved in gene electrotransfer have been identified: electropermeabilization of the cell membrane, contact of the plasmid with the cell membrane, translocation across the membrane followed by movement through the cytoplasm, entering the nucleus and gene expression (Escoffre et al. 2009).

Furthermore, several in vivo studies have shown the importance of electrophoretic movement of DNA during the pulses (Bureau et al. 2000; Satkauskas et al. 2002, 2005; Andre et al. 2008) by showing that using a combination of high-voltage (HV) and low-voltage (LV) pulses enhances DNA electrotransfer. It was suggested that HV electric pulses destabilize the cell membrane and enable DNA insertion into the cell membrane, whereas LV pulses produce electrophoretic force which drags negatively charged DNA molecules toward and/or into the cell. However, with the exception of two studies (Klenchin et al. 1991; Sukharev et al. 1992) in which the authors first used an HV + LV combination of pulses, the in vivo observertions have not been confirmed in vitro.

Recently, we demonstrated (Kandušer et al. 2009) that HV + LV pulses increase transfection efficiency only in conditions where plasmid concentration is relatively low, i.e., for suboptimal plasmid concentrations, which is typical for the in vivo environment. Since poor plasmid mobility in tissues is one of the major barriers to the use of gene electrotransfer for gene therapy (Zaharoff et al. 2002; Cemazar et al. 2006; Henshaw et al. 2007), we present here an in vitro study where we tested the hypothesis that adding an LV pulse before the HV pulse could increase tranfection efficiency, which is of interest also for in vivo application of gene electrotransfer. So far only a single study done in vivo used such a pulsing protocol (Bureau et al. 2000); however, the delay between the LV and HV pulses was relatively long (around 2 s). For this reason, we designed a special prototype of electroporator which enables use of different combinations of HV and LV pulses. Furthermore, since we were able to switch the time course of HV and LV pulses, it enabled us to further test a different hypothesis, that LV provides electrophoretic force which (1) enables efficient contact of the DNA molecule with the cell membrane, (2) helps insertion of DNA into permeabilized cell membrane, (3) drags DNA across the permeabilized cell membrane and (4) combinations of these possibilities.

Materials and Methods

Cells

Chinese hamster ovary (CHO) cells (European Collection of Cell Cultures, Salisbury, UK) were grown as a monolayer culture in F12 HAM nutrient mixture (GIBCO, Grand Island, NY) supplemented with 2 mM glutamine, 10% fetal bovine serum (Sigma-Aldrich Chemie, Deisenhofen, Germany) and antibiotics at 37°C in a humidified 5% CO_2 atmosphere in an incubator.

Electrotransfection Protocol

To generate electric pulses, a new prototype was developed which enabled us to apply only HV pulses, only LV pulses and a combination of both pulses, either HV pulses followed by LV pulse (HV + LV) or LV pulse followed by HV pulses (LV + HV). A pair of parallel wire electrodes was used, with the distance, d, between them being 4 mm. In pulsing protocols we used four HV pulses of 200 μs duration with amplitude $E_{HV} = 1.0$ kV/cm ($U = 400$ V) and/or one LV pulse of duration 100 ms with $E_{LV} = 0.075$ kV/cm ($U = 30$ V). The delay (lag) between HV and LV or LV and HV pulses was in the first set of experiments set to 20 ms. In further experiments, where the effect of the lag between HV and LV pulses was analyzed, the lags were 20 ms, 100 ms and 5 s.

Experiments

Plasmid DNA pEGFP-N1, purified with the Endofree Plasmid mega kit (Qiagen, Valencia City, CA) coding for green fluorescent protein (GFP), was used to analyze the efficiency of gene electrotransfer. Experiments were performed on plated cells seeded in 24-well plates with a concentration of 5×10^4 cells/well. On the day of the experiment, the growth medium was removed and replaced with the mixture of plasmid DNA and iso-osmolar pulsing buffer (pH 7.4, 10 mM Na_2HPO_4/NaH_2PO_4, 1 mM $MgCl_2$ and 250 mM sucrose). Final concentrations of plasmid were 1, 5 and 10 μg/ml. The optimal concentration (10 μg/ml) was determined experimentally as the concentration above which the increase in concentration of the plasmid did not further increase the transfection efficiency. We incubated cells in the pulsing buffer for 2–3 min at room temperature (22°C). Then, different pulsing combinations were delivered. Treated cells were incubated for 5 min at 37°C and then grown for 24 h in cell culture medium at 37°C in a humidified 5% CO_2 atmosphere in an incubator. Efficiency of transfection was determined by fluorescence microscopy (Axiovert 200; Zeiss, Oberkochen, Germany). Images were recorded using the MetaMorph imaging system (Visitron,

Puchheim, Germany), and at least five phase-contrast and five fluorescence images were acquired for each parameter. Cells were counted manually, and the transfection efficiency was determined by the ratio between the number of fluorescent cells and the number of cells counted under the phase contrast. In the same experiments, viability of cells was obtained as the ratio between the number of viable cells in the treated sample and the number of viable cells in the control sample. At least five independent experiments were performed for each parameter, and the results are presented as mean values ± standard deviation. The data were statistically analyzed by one-way ANOVA, and the differences among pulsing protocols were tested by Bonferroni's *t*-test.

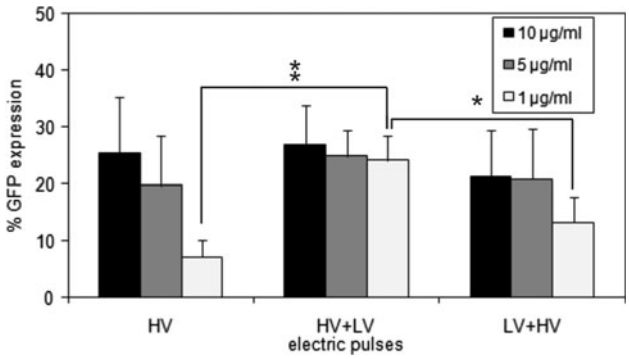

Fig. 1 Effect of electric pulse protocols on the percentage of CHO cells expressing GFP at different plasmid concentrations. Each bar is a mean of five independent experiments ± standard deviation, ** $P < 0.001$ and * $P < 0.01$

Results and Discussion

Relatively poor efficiency of gene electrotransfer in vivo is considered to be the major obstacle toward application of electroporation for efficient gene therapy. Several studies have shown that poor plasmid mobility in tissues is one of the major barriers to delivering DNA into cells with electric pulses (Henshaw et al. 2007). In general, it was demonstrated that longer pulses (millisecond duration) were more efficient compared to shorter ones (microsecond) for gene transfection in vivo. Furthermore, it was shown that use of specific combinations of HV and LV pulses increased the efficiency of gene electrotransfer (Satkauskas et al. 2005). It was suggested that LV pulses provide electrophoretic force that (1) drags negatively charged DNA and brings it into contact with the cell membrane, (2) increases insertion of DNA into the permeabilized membrane and (3) drags DNA across the cell membrane (translocation).

We used different sequences of HV and LV pulses in order to understand the role of electrophoretic force in electrotransfection efficiency. For this reason, we designed a special prototype of electroporator which enabled us to apply different combinations of HV and LV pulses. We determined gene electrotransfer efficiency in vitro for different pulsing combinations using optimal (in vitro) plasmid DNA concentrations as well as relatively low suboptimal concentrations. Furthermore, we also analyzed the effect of lag (delay) between HV and LV pulses for the lowest (suboptimal) plasmid concentration.

In Fig. 1 the effect of plasmid concentrations on GFP expression for different electric pulse protocols is presented. It can be seen that when only HV pulses are applied, the gene expression drops significantly ($P \leq 0.05$) when the plasmid concentration is reduced from 10 to 1 µg/ml. For the highest plasmid concentration (10 µg/ml) there are no significant differences among different pulsing protocols, transfection efficiency being 20–27%. However, for suboptimal plasmid concentration (1 µg/ml) the HV + LV pulsing protocol is statistically more efficient ($P \leq 0.001$) compared to using only HV pulses. Furthermore, when the order of application of HV and LV pulses is reversed and the LV pulse is applied before the HV pulses, GFP expression is slightly, but not significantly, increased ($P = 0.09$) at suboptimal concentration with respect to HV treatment only. The reversed combination of LV and HV pulses (LV + HV) at 1 µg/ml resulted in significantly lower ($P = 0.003$) expression compared to HV + LV. These trends can be observed also at 5 µg/ml plasmid concentration, although they are not statistically significant. Almost no transfection (below 1%) was obtained for only LV pulse (results not shown).

Since the only statistically significant differences were found between the HV + LV and only HV pulsing protocols at the suboptimal plasmid concentration, we analyzed the effect of lag between the application of HV and LV pulses for plasmid concentration of 1 µg/ml (Fig. 2). We observed no statistical differences for different lags when changing lag from 20 ms to 5 s, which is in agreement with previously published in vivo results (Satkauskas et al. 2002).

In all the experiments the viability of the treated cells was not significantly reduced by any of the pulsing protocols used and was maintained at around 80% (results not shown). Our results confirm that, as already reported in our previous study (Kandušer et al. 2009), the combination of HV and LV pulses at suboptimal concentration of DNA (1 µg/ml) significantly increased gene expression ($P < 0.001$) compared to application of only HV pulses, as shown in Fig. 1. Furthermore, when analyzing the concentration dependence as shown in Fig. 1, it can be clearly seen that for HV pulses decreasing plasmid concentration drastically decreased transfection efficiency, while for the combination of HV and LV it stays at the same level.

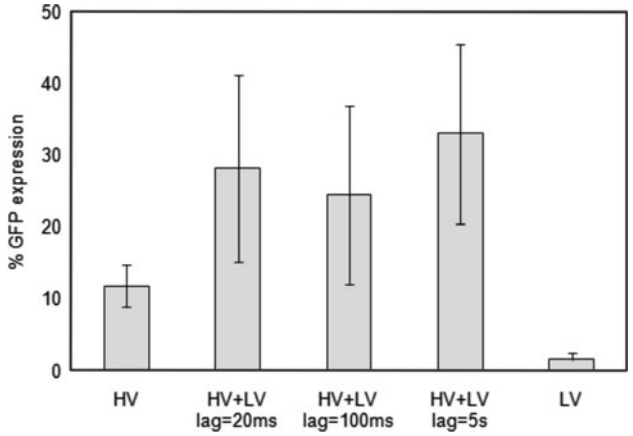

Fig. 2 Effect of lag (delay) between HV and LV pulses on transfection efficiency, percentage of cells expressing GFP at suboptimal plasmid concentration 1 μg/ml. Each bar is a mean of four independent experiments ± standard deviation

Interestingly, the combination of LV + HV pulses slightly increased GFP expression compared to application of only HV pulses, even though we could not confirm this statistically. This observation is similar to that from an in vivo study (Bureau et al. 2000), where LV pulses applied before HV pulses slightly increased gene transfection even though they used a longer lag between LV and HV pulses (around 2 s).

It appears that LV pulse before the HV pulses has also some positive effect on transfection efficiency. Our hypothesis is that if the LV pulse is applied before HV pulses, electrophoretic force drags some additional DNA into contact with the cell membrane. However, since the HV + LV combination is significantly more effective than the LV + HV combination, our results also suggest that adding an LV pulse after HV pulses contributes to increased DNA insertion and/or translocation across the cell membrane and that this effect appears to be dominant. It appears that the contributing effect of the LV pulse is present on a time scale of several seconds (see Fig. 2), suggesting that insertion/translocation in/across the permeabilized membrane is a relatively slow process, in agreement with other studies (Golzio et al. 2002).

To conclude, in this study we demonstrated that HV + LV pulses increase transfection only in conditions where plasmid concentration is low; therefore, suboptimal plasmid concentrations have to be used in order to mimic in vivo conditions. Only then electrophoretic force of the LV pulse has an effect on final transfection efficiency, the effect being much more pronounced for the HV + LV combination, while for the reversed sequence (LV + HV) there is only a minor effect of the LV pulse. For the highest plasmid concentration no added effects of the LV pulse were observed. It appears that there are different contributing effects of the LV pulse: (1) to a minor extent electrophoretically increased contact of DNA with the membrane and (2) to a larger extent increased insertion of DNA into permeabilized cell membrane and/or translocation across the cell membrane.

Importantly, even though there was only small improvement of transfection efficiency (statistically not significant) for the LV + HV protocol, we suggest that use of the LV + HV combination could improve transfection efficiency in vivo where both low plasmid concentration and poor plasmid mobility are limiting factors. One can also speculate that using an LV + HV + LV combination could improve transfection efficiency in vivo.

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