Bioelectrochemistry 103 (2015) 111–119

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Inhibitor of endocytosis impairs gene electrotransfer to mouse muscle in vivo

Bostjan Markelc [a], Eva Skvarca [a], Tanja Dolinsek [a], Veronika Prevodnik Kloboves [b], Andrej Coer [c], Gregor Sersa [a,*], Maja Cemazar [a,c,**]

[a] Department of Experimental Oncology, Institute of Oncology Ljubljana, Zaloska 2, SI-1000 Ljubljana, Slovenia
[b] Department of Cytopathology, Institute of Oncology Ljubljana, Zaloska 2, SI-1000 Ljubljana, Slovenia
[c] Department of Natural and Medical Subjects, Faculty of Health Sciences, University of Primorska, Polje 42, SI-6310 Izola, Slovenia

ARTICLE INFO

Article history:
Received 27 January 2014
Received in revised form 1 August 2014
Accepted 12 August 2014
Available online 29 August 2014

Keywords:
Electropermeabilization
Gene electrotransfer
Plasmid DNA
Endocytosis
Muscle

ABSTRACT

Application of electric pulses (electroporation/electropermeabilization) is an effective method for gene transfer (i.e. gene electrotransfer (GET)) *in vitro* and *in vivo*. Currently, the mechanisms by which the DNA enters the cell are not yet fully understood. Experimental evidence is building up that endocytosis is the main mechanism by which the DNA, which is later expressed, enters the cell. Therefore the aim of our study was to elucidate whether inhibitors of endocytosis, methyl-β-cyclodextrin (MβCD), Concanavalin A (ConA) and Dynasore, can impair the transfection efficacy of GET *in vitro* in B16F1 murine melanoma and *in vivo* in *m. tibialis cranialis* in mice. We show that MβCD – general inhibitor of endocytosis – can almost prevent GET of EGFP-N1 plasmid *in vitro*, that ConA – inhibitor of clathrin mediated endocytosis – also abrogates GET but to a lesser extent, and when using Dynasore – reversible inhibitor of dynamin – there is no effect on GET efficacy, if endocytosis is blocked for only 5 min after GET. Moreover, MβCD also reduced GET efficacy *in vivo* in *m. tibialis cranialis* and this effect was long lasting. The results of this study show that endocytosis is probably the main mechanism of entrance of DNA after GET *in vitro* and also *in vivo*.

1. Introduction

Gene transfer into cells or tissues by the application of electric pulses (i.e. gene electrotransfer (GET)) is becoming increasingly attractive method for transfection due to its ease of use and good transfection efficiency, especially *in vivo* [1–7]. Electrotransfer of plasmid DNA by electroporation/electropermeabilization *in vitro* has been discovered in 1980s and has already been successfully used in a variety of tissue types *in vivo* including the skin, kidney, liver, testis, brain, cartilage, arteries, prostate, cornea and skeletal muscle [8–10]. Moreover, reports from several clinical trials using GET have already been published and showed the safety and efficacy of the method for the treatment of cancer as well as for DNA vaccination [11–13].

Conversely, despite the success the method has achieved its underlying mechanisms, which are controlling the transfer of plasmid DNA across the cell membrane, are poorly understood [14–18]. It is known that the mechanism of entrance into the cell differs for small molecules such as dyes and small RNA molecules (siRNA, miRNA, etc.) and for large DNA molecules such as plasmid DNA [18,19]. Entrance of small molecules into the cells is driven by electrophoretic force during the pulse application across the permeabilized cell membrane, probably through "electropores" that are induced in the membrane due to the application of electric pulses [20–24]. Small molecules can also diffuse through the same electropores for several minutes after the pulse application, whereas the plasmid DNA has to be present at the time of application of electric pulses in order to cross the cell membrane; however, the mode of entrance for large DNA molecules (plasmid DNA) into the cells is still in debate [16,18]. Currently there is a consensus that gene electrotransfer is a multistep process where the application of electric pulses is necessary for the creation of DNA complexes with cell membranes, so called spots or aggregates [25–27]. These aggregates are formed only on the side of the cell facing the negative electrode, due to the negatively charged DNA, and, if more than one pulse is applied, these aggregates are formed during the first applied electric pulse [25–27]. After the formation of the aggregates the electrophoretic force of the applied electric pulses pushes the free DNA towards the already formed aggregates and increases the quantity of the DNA that is bound in them [25–27]. The DNA inside the cells could be detected in the cytosol only several minutes after the application of electric

* Corresponding author. Tel.: +386 1 5879 434; fax: +386 1 5979 434.
** Correspondence to: M. Cemazar, Department of Experimental Oncology, Institute of Oncology Ljubljana, Zaloska 2, SI-1000 Ljubljana, Slovenia. Tel.: +386 1 5879 544; fax: +386 1 5979 434.
E-mail addresses: gsersa@onko-i.si (G. Sersa), mcemazar@onko-i.si (M. Cemazar).

http://dx.doi.org/10.1016/j.bioelechem.2014.08.020
1567-5394/

pulses either in the form of DNA aggregates or as free DNA suggesting that DNA could cross the membrane through electropores or via endocytosis [26,28]. There is some evidence that diffusion of DNA through the electropores, as suggested in the standard electroporation theory, is feasible [18,26]; however, due to the size of the DNA and its relatively short half-life in the cytosol [29–31] a more likely path is the intake of DNA bound in the aggregates by endocytosis. Recent *in vitro* studies on Chinese Hamster Ovary (CHO) cells have shown that up to ~75% of the DNA aggregates that are detected in the cytosol within minutes after the application of electric pulses enter the cell through endocytosis [28,32]. Once inside the cell, these aggregates are actively transported towards the nucleus by cytoskeleton, primarily with microtubules [33,34]. The onset of transgene expression is then observed 3 h after the exposure of electric pulses [26].

However, there are still uncertainties whether the expression of transgene is due to the DNA which is taken up by endocytosis or due to the DNA that entered the cell through electropores and to what extent each of these processes contributes to the transfection efficiency of GET [15,25,27,28,35–38].

Therefore, to determine to what extent the transfection efficiency of GET is controlled by the uptake of DNA by endocytosis we used 3 different inhibitors of endocytosis and studied their effect on the transfection efficiency of GET in murine melanoma cells *in vitro* and in *m. tibialis cranialis in vivo* in mice. The obtained data provides further proof that transfection efficiency of GET strongly depends on the endocytic uptake of DNA *in vitro* as well as *in vivo*.

2. Materials and methods

2.1. Study design

To test whether endocytosis is involved in electrotransfer of plasmid DNA we tested the effect of three different inhibitors of endocytosis on GET efficacy of EGFP-N1 plasmid DNA *in vitro* in B16F1 murine melanoma cells and in *in vivo* in mouse muscle *m. tibialis cranialis*. First, we determined the cytotoxicity of all three inhibitors *in vitro* at day 3 after the treatment. Second, we evaluated the effect of chosen concentrations of inhibitors on GET efficacy *in vitro* by flow cytometry. Third, the effectiveness of endocytosis inhibition by the inhibitors was confirmed with the reduction of FITC-transferrin uptake determined by flow cytometry. Fourth, we determined the effect of one of the inhibitors of endocytosis on the GET efficacy of EGFP-N1 plasmid DNA *in vivo* in mouse muscle *m. tibialis cranialis* with fluorescence imaging of the transfected muscle at different days after GET and determined the effect of the treatment by the histological properties of the transfected muscle.

2.2. Cell line and animals

Murine melanoma cell line B16F1 (ATCC, Rockville, USA) was cultured in advanced minimum essential medium (MEM, Gibco, Life Technologies, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS, Gibco, Life Technologies, Grand Island, NY, USA), 10 mM L-glutamine (GlutaMAX, Gibco, Life Technologies, Paisley, UK), 100 U/ml penicillin (Grünenthal, Aachen, DE) and 50 mg/ml gentamicin (Krka, Novo mesto, Slovenia) in a 5% CO_2 humidified incubator at 37 °C.

In the experiments, female C57Bl/6 mice were used (Institute of Pathology, Faculty of Medicine, University of Ljubljana, Slovenia). Experiments were performed in accordance with the guidelines for animal experiments of the European Union directives and the permission from the Ministry of Agriculture and the Environment of the Republic of Slovenia (Permission no.: 34401-4/2012/2). Mice were kept under specific pathogen-free conditions at a constant room temperature (21 °C) and a 12-h light/dark cycle. Food and water were provided ad libitum. Animals were subjected to an adaptation period of 14 days before experiments. Experimental groups consisted of 6 animals.

2.3. Reagents

The inhibitors of endocytosis methyl-β-cyclodextrin (MβCD), Concanavalin A (ConA) and Dynasore were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, USA). Before every experiment a fresh stock solution of inhibitors was prepared in 1× PBS buffer. The stock solutions of inhibitors (MβCD — 30 mM, ConA — 9.61 mM (1 mg/ml), Dynasore — 0.25 mM) were then diluted to working concentrations directly on the day of experiments as described below. FITC-transferrin (Life Technologies, Carlsbad, California) was resuspended in 1× PBS to a stock concentration of 5 mg/ml. Paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) was diluted to the working concentration of 4% in 1× PBS.

Plasmid pEGFP-N1 (Clontech Laboratories Inc., Mountain View, USA), encoding enhanced green fluorescent protein (pEGFP-N1), was prepared from *Escherichia coli* cultures using the Qiagen Endo-Free Plasmid Mega Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions and diluted in H_2O to the working concentration of 1 μg/μl. In all *in vitro* experiments 10 μg of DNA/sample was used and in all *in vivo* experiments 20 μg of DNA/mouse was used.

2.4. Treatment of cells with inhibitors of endocytosis

The B16F1 melanoma cells grown as monolayer were collected and a cell suspension with a concentration of 2×10^6 cells/ml was prepared in a MEM without FBS and antibiotics. Then 0.5 ml of the prepared cell suspension was mixed with different volumes of 1× PBS and the stock solution of inhibitors of endocytosis in order to obtain final concentration of the inhibitors ranging from 1.25–20 mM for MβCD, 0.024–3.85 mM (2.5–400 μg/ml) for ConA and 0.015–0.080 mM for Dynasore. The total volumes of the prepared mixture were kept constant for each inhibitor; 5 ml for MβCD and 1.5 ml for ConA and Dynasore. Next, the cells were incubated for 30 min in a humidified incubator at 37 °C and 5% CO_2 and after the incubation 10 ml of electroporation buffer (EP buffer: 125 mM sucrose, 10 mM K_2HPO_4, 2.5 mM KH_2PO_4, 2 mM $MgCl_2 \times 6\ H_2O$) was added. The cells were then centrifuged and the supernatant was removed. The remaining pellet was then resuspended in MEM with FBS and antibiotics or EP buffer, depending on the subsequent experiments as described below.

2.5. Cell viability

To determine the cell viability, the treated cells (1000 cells in 100 μl ml of MEM with FBS and antibiotics) were plated on 96-well plates (Corning Inc., Corning, NY, USA). The cells were incubated at 37 °C in a 5% CO_2 humidified incubator. At day 3 after the treatment, the Presto Blue assay (Life Technologies) was performed. Ten microliter of Presto Blue reagent was added to each well and then the fluorescence intensity in the well was measured after 20 min of incubation at 37 °C in a 5% CO_2 humidified incubator with a microplate reader (Infinite 200, Tecan, Männedorf, Switzerland). Cell viability was expressed as the fraction of viable cells normalized to the control group, which was not treated with inhibitors.

2.6. Endocytosis of transferrin

To determine the endocytosis of transferrin the treated cells were resuspended in 500 μl of phenol free MEM with FBS, antibiotics and 2 μl of FITC-transferrin stock solution. This was followed by 20 min incubation in a 5% CO_2 humidified incubator at 37 °C, after which 1 ml of 1× PBS was added to the mixture followed by centrifugation. The supernatant was removed and the pellet was resuspended in 1 ml of 4% paraformaldehyde. The cells were incubated with 4% paraformaldehyde for 20 min to fix them, then the cells were centrifuged, the supernatant was removed, and the pellet was resuspended in 500 μl of citrate buffer (25 mM citric acid, 24.5 mM sodium citrate, 280 mM sucrose) with